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Migration of surface-associated microbial communities in spaceflight habitats

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Abbreviations:

CFU: Colony forming unit

CLSM: Confocal Laser Scanning Microscopy

ECLSS: Environmental Control and Life Support System

e-DNA: extracellular DNA

EPS: Extracellular polymeric substance

ESA: European Space Agency

ESKAPE: acronym describing six highly virulent and antibiotic-resistant bacterial pathogens of major interest in human health including (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp.)*

EVA: extra-vehicular activity

FGB: Functional Cargo Module

ISS: International Space Station

LSMMG: low-sheared modelled microgravity

NASA: National Aeronautics and Space Administration (America's civil space program)

OMV: outer membrane vesicles.

PRW: Persistence Random Walk model

QS: Quorum-sensing

RNA-seq: RNA-sequencing for high-throughput transcriptomics analysis

SUS: super-hydrophilic and underwater superoleophobic material

T4P: type-IV-pili

WPA: Water Process Assembly

WRS: Water Recovery System

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Abstract

Astronauts are spending longer periods locked up in ships or stations for scientific and exploration spatial missions. The International Space Station (ISS) has been inhabited continuously for more than 20 years and the duration of space stays by crews could lengthen with the objectives of human presence on the moon and Mars. If the environment of these space habitats is designed for the comfort of astronauts, it is also conducive to other forms of life such as embarked microorganisms. The latter, most often associated with surfaces in the form of biofilm, have been implicated in significant degradation of the functionality of pieces of equipment in space habitats. The most recent research suggests that microgravity could increase the persistence, resistance and virulence of pathogenic microorganisms detected in these communities, endangering the health of astronauts and potentially jeopardizing long-duration manned missions. In this review, we describe the mechanisms and dynamics of installation and propagation of these microbial communities associated with surfaces (*spatial migration*), as well as long-term processes of adaptation and evolution in these extreme environments (*phenotypic and genetic migration*), with special reference to human health. We also discuss the means of control envisaged to allow a lasting cohabitation between these vibrant microscopic passengers and the astronauts.

Keywords: Biofilm, space flight, microgravity, transcriptomic, adaptation, evolution, control.

1. Introduction

Human space exploration presents many challenges for space agencies, habitability engineers and microbiologists, especially in the upcoming new era of human expansion in the universe, such as future space travel to Mars. Internal spacecraft must provide safe levels of biological, chemical and physical parameters to astronauts. Space spaceships and stations are closed systems inhabited by

microorganisms that originate from different sources including the initial contamination of space flight materials during manufacturing and assembly, the delivery of supplies, the automicroflora of the crew and other biological materials present on board [1]. In space habitats, environmental conditions (gas composition, pressure, temperature, and humidity) are set to the comfort of astronauts (e.g. 22°C, 6O% of relative humidity in the International Space Station (ISS) [2]) and are also favourable to other forms of terrestrial life such as embarked bacteria, yeasts, moulds or viruses. Microorganisms are ubiquitous and will in general accompany human-inhabited spacecraft without imposing dramatic safety concerns. However, if biological contamination were to reach unacceptable levels or if it contains microorganisms at risk (for astronauts and their equipment), long-term human space flights could be jeopardized. In these environments, most microorganisms are associated with surfaces in spatially organised microbial communities termed biofilms which can be defined as surface-associated communities of microorganisms embedded in self-produced extracellular polymeric substances (EPS) [3]. This microbial mode of life significantly differs from free planktonic cultures in homogeneous Newtonian liquid environments. Cells in a multilayered biofilm experience a diversity of local microenvironments within the matrix and intensive cell-to-cell interactions with other community members. Biofilm structures are associated with emerging community functions such as a dramatic tolerance to the action of antimicrobials [4]. Important material degradation associated with microbial biofilm development has been reported in several space stations (**Figure 1**). The affected parts were for example piping and equipment behind panels, headphone of extra-vehicular activity (EVA) suit, thermal control system, rubber of hatch locks, electrical connectors, radiators, air conditioning, water recycling systems and oxygen electrolysis block [5]. The microbially-induced degradation of a navigation window was associated with the presence of *Bacillus polymira, Penicillium rubens and Aspergfilus sp*. [5]. On the ISS, the most severely affected units are wastewater collection reservoirs, also known as the Water Process Assembly (WPA) of the Water Recovery System (WRS) which is part of the Environmental Control and Life Support System (ECLSS). For WPA the most common microbial organisms isolated are *Ralstonia picketii, Bulkholderia sp.* and *Cupriavidus metallidurans* [6]. Biofilm formation is critical in any spacecraft

system, however, it is of utmost relevance when it affects ECLSS, given the relevance of this system to the health of the crew.

Lessons learnt in previous space missions suggest that prevention of microbiological problems is preferred over mitigation, and prevention steps must be taken into consideration from the very early design phase. Requirements to control free water from condensate, hygiene activities, humidity, condensate and other releases must be included in every spacecraft system development. Water is one of the main driving elements for microbial outgrowth and its accumulation must be avoided and controlled either by hygienic design or by water processing techniques, such as thermal inactivation, filtration and biocide treatments. Furthermore, the materials selected must not promote microbial growth and system design must include the onboard capability to achieve recovery of the system from microbial contamination. Robust housekeeping procedures that include periodic cleaning and disinfection are required. In addition, routine and systematic microbial monitoring of surfaces, air, and water using culture-based techniques is conducted by each space agency [7]. Monitoring includes two levels of sample analysis. The first level corresponds to a real-time assessment of the microbial load and dynamics on the basis of total microbial counts. The second level is the ground-based assessment of species composition, properties, and characteristics of archived samples which were collected in-flight, as well as samples that are collected 1-2 days before crew return [8]. However, culture-based analysis limits the understanding of the diversity of microorganisms in space habitats as only a small fraction of organisms can be cultured under standard laboratory conditions [9]. Implementing molecular methods on board the spaceship will enable the identification and quantification of both culturable and unculturable organisms providing a more in-depth assessment of the microbial population and density [10]. This is of utmost importance considering the long-term human space exploration and associated protection of planet contamination [11]. On the International Space Station, air cleanliness is ensured through the implementation of the Potok system [12]. The microbe-killing principle of Potok is through the use of an electrical field of alternating polarity with fine electrostatic filtration of microbe decomposition products. In the framework of ESA's Microbial Detection in Air System for Space (MiDASS) project a miniaturised automated system was developed for the sampling and monitoring of the microbiological quality of air, surfaces, and also potentially

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water and food [13]. The system comprises two modules: sample preparation with nucleic acids extraction, and module with nucleic acids amplification and detection [14].

In regards to biocontamination analysis onboard spacecraft, Nokivoka et al. [15] reported that in the Mir orbital station, bacterial concentration in airborne contamination was below 5 x 10^2 Colony Forming Unity (CFU)/m^3 where bacterial genera *Staphylococcus sp., Corynebacterium sp.,* and *Bacillus* species were dominant. The concentration of airborne fungi fluctuated between 2 and 5 x 10^4 CFU/m^3, with *Penicillium* and *Aspergillus* as the dominant genera. Contamination levels of surfaces and equipment on board were also variable, with bacterial and fungal concentrations between 10 and 10^5 CFU cm^2, where the dominant bacterial and fungal genera were closed as for airborne contamination. Dominant opportunistic pathogenic bacteria were also identified, compiling among others *Flavobacterium meningosepticum, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Staphylococcus* sp, etc. Some of these microorganisms have been associated with infectious diseases in respiratory organs and the digestive tract. Biocontaminants isolated on board the Mir orbital station are to a great extent comparable [16] to the results obtained from the ISS [17] [18] [19] [20] [21] [22] [23].

These reports highlight that the microbiota in inhabited space crafts is mainly associated with surfaces (often in contact with the crew) and dominated by human automicrobiota, including pathogenic species. Specific concerns about detected pathogens were pinpointed recently including a high prevalence of antibioresistant isolates, many of them listed in the ESKAPE list (the six most highly virulent and antibiotic-resistant human bacterial pathogens: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp*) [26] [27] [28]. 76% of isolates from the Russian segment on the ISS show resistance to one or more antibiotics, questioning the evolution of this microbiota and its interactions with astronauts in long-term missions [27].

Recent fundamental studies on bacterial biofilms exposed to microgravity also pinpointed specific traits of serious concerns for the crew safety in long-term spaceflight missions: i) an important global regulator involved in the pathogenesis of *Salmonella typhimurium* was shown to be highly overexpressed aboard the space shuttle mission STS-115 compared to the ground control condition,

suggesting hypervirulent physiology of this pathogen under microgravity exposure [29], ii) *Pseudomonas aeruginosa* cultivated in microgravity condition (space shuttle missions STS-132 and STS-135) generate more biomass and adopted a unique canopy-like biofilm structure instead of the flat architecture observed in terrestrial conditions [30]. The impact of this specific structure on pathogens persistency and virulence is not elucidated yet, but the knowledge acquired on the links between biofilm architecture and their functions would suggest specific adaptive processes in these biological systems exposed to microgravity [31] [32] [33]. To illustrate the impact of microgravity exposure on *P. aeruginosa* cell microenvironments, we computed a reaction-diffusion model from real microscopic images from [30] showing shaper gradients of nutrients for biofilm cells exposed to microgravity (**Figure 2**).

Altogether, the accumulation of data from spaceflight habitats and microgravity exposition of microorganisms suggest that biofilms' emerging properties make them an essential issue to take into account in long-duration space flights, as they could increase the risk and severity of microbial infection [34]. The objective of this review is to consider not only the mature biofilm traits in long-term spaceflight habitats, but the whole dynamic of the biosystem, including the populations of cells migrating on the surface to initiate new biofilms, the populations migrating inside a biofilm matrix and the populations emigrating from a biofilm to propagate the community (**Figure 3**). We will also discuss the phenotypic and genetic *migration* of these vibrant surface communities that are continuously adapting and evolving to the specific conditions encountered in these biotopes, with special reference to crew health, and discuss envision control strategies.

2. The mechanisms of microbial migration on surfaces

Microorganisms can move across their environment through passive means such as colloidal particles, but also through highly sophisticated and tightly regulated mechanisms involving specific appendages

or cellular processes. Each cycle starts with the transport of the organisms from bulk to the host surface.

2.1 The effect of gravity on bulk microbial transport

In general, the bulk transport of microbes occurs either in gas or liquid phases. In the first case (gas) there are two possibilities for the microenvironment of microbes depending on the cell size [35]: (i) aerosols for cells < 1μm such as bacterial spores or viruses, (ii) suspended droplets for cell units with size > 1 μm. The transported aerosol units could have sizes quite similar to the cell size whereas several microbes could exist in a liquid droplet of size fairly larger than 1 μm [36] [37]. From now on, both types of transported units are called droplets (considering their varying amount of liquid). In case of favourable droplet/substrate interactions, droplet deposits through Brownian motion (i.e., diffusion) on a substrate, creating a local droplet concentration deficiency in the gas phase. The combination of the concentration gradient imposed by diffusion and of the gas flow transferring the droplet constitutes the convective-diffusion droplet deposition mechanism. The deposition is affected also by other mechanisms that lead to deviation of the droplet motion from the gas motion. Such mechanisms are the (droplet) inertia plus several external force fields like the gravitational one (leading to sedimentation), the electrical one (leading to electrical precipitation) and the thermal one (leading to thermophoresis) [38]. The relative contribution of gravity and Brownian diffusion to the deposition rate is described by the dimensionless number $N_G=4\pi r^4(\rho_p-\rho)g/(3k_BT)$ [39] where r is the droplet radius, g is the gravitational acceleration, ρ_p, ρ are the droplet and gas density respectively, k_B is the Boltzmann constant and T is the temperature. Introduction in the equation for N_G of representative sizes yields that for microbes of type (i) gravity plays a negligible to small role in the deposition efficiency (depending on microbe size). On the contrary, under terrestrial conditions, gravity completely dictates the deposition behaviour of microbes of type (ii).

The second way of bulk transport is inside the liquid phase. It is noted that motility and several types of taxi motion may affect the bacteria transport in the bulk. In this section, only the passive bulk

transport is examined, see section 2.2 for microbial active mechanisms. The deposition rate of microbes depends not only on bulk transport but also on physicochemical interactions between the microbial cell and the substrate. These thermodynamic interactions have been described by the general colloidal model proposed by Derjaguin−Landau−Verwey−Overbeek (DLVO) and reviewed extensively elsewhere [40]. Some specific domains where the liquid phase bulk transport of microbes is of paramount importance are wastewater and potable water pipe networks, groundwater flows, deep bed filtration, and reverse osmosis membrane biofouling. Each particular application determines the geometry of the involved flow field (pipe, structured or unstructured porous media). In general, bulk transport is considered quite important for the generation of a biofilm which refers actually to a biofilm seeding process *i.e.*, transport and deposition of microbes on a clean surface. On the other hand, bulk transport is not so important for the subsequent stage of biofilm growth (where sometimes [41] it is even ignored) which is a very complex process driven by its inherent dynamics. So after the aforementioned clarifications, it is apparent that the passive motion of non-motile microorganisms toward a surface is driven by similar processes to that of inert colloidal particles. There is an enormous amount of studies on the deposition of colloidal particles on surfaces [42]. The main bulk transport mechanism (similar to that discussed before in the context of aerosols) is the combination of diffusion (Brownian motion) and motion within the fluid (*i.e.* convective-diffusion mechanism). Further deposition can occur by causes leading to the deviation between microbe and fluid streamlines. Such causes are microbe inertia and gravity. The inertia effect is associated with the Stokes number which is proportional to the square of particle size. Although no detailed calculation of this number can be performed, as it depends on specific flow velocity and size scales, it can be argued that inertia is negligible for particle size of a few microns. An additional deposition mechanism can be found under the name "interception" [43]. This is simply due to the combination of the flow field and of finite particle size. The relative effect of gravity and diffusion on deposition is described by the number N_G which is discussed before (where ρ_p, ρ are the microbe and the liquid density, respectively). Knowledge of the microbe density is required in order to calculate N_G. Several values between 1.03 g/cm^3 and 1.14 g/cm^3 have been reported in the literature [44]. An average value of 1.085 g/cm^3 will be used herein for the calculations. The value of N_G is 0.053 for r=0.5 μm and 4.4 for

r=1.5 μm which means that gravity has only a small contribution in the deposition for a microbe size of 1 μm but dominates it for a microbe size of 3 μm. Another interesting issue is that most microbes are not perfect spheres (ovoccoids, bacilli, filaments…). This makes their interaction with the flow field very complex. Usually, a spheroidal shape is considered [45] for which several hydrodynamic theoretical approaches can be found in the literature [46]. The shape effect typically leads to larger deposition rates compared to the theories based on a spherical shape. The above order of magnitude analysis has been supported by experimental studies on microbe deposition. In [47] it is argued that the measured deposition rate is higher than the theoretical one based on convective diffusion, due to gravitational contribution. More significantly, in [48] it was shown that for microbes with an aspect ratio of 1.91 and an equivalent diameter of 1.7 μm the deposition rate strongly depends on the direction of gravity with respect to the surface of deposition. In addition, the deposition rate differs from that taken in the absence of gravitational contribution (achieved by density matching). Finally, it is found that the microbe deposition rate is larger than that of spherical colloidal particles with a diameter close to the equivalent diameter of the microbes (attributed to their non-spherical shape discussed above). It is noted that in [48] the definition of N_G is somewhat different than the original one in [39] having 2 as a constant parameter in the denominator instead of the correct 3. On obstacles, the interplay between fluid shear and microbial motility allows the accumulation of elongated bacteria in unattainable locations for passive particles [49]. The effect of gravity on microbe deposition has also been indirectly confirmed experimentally by observing the spatial distribution of microbes bulk concentration [50]. Furthermore, the use of fluorescence imaging in [50] allowed the measurement of increased deposition rates along with the flow. This behaviour is totally opposite to the one predicted by convective-diffusion, but is in accordance with a sedimentation-based model. In summary, it appears that gravity may be quite important at least at the stage of biofilm seeding and its absence would certainly yield different results in many cases. However, it must be stressed that increased deposition rates due to microbe-specific mechanisms (motility, several taxis) may reduce the contribution of gravity to deposition.

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In addition to these passive movements, a large proportion of microorganisms can actively propel themselves into an environment governed by viscosity using different appendices. Microorganisms motion can be achieved by different mechanisms: swimming, swarming, gliding, twitching, and sliding [51]. Regardless of the type of motility machinery that is employed, most motile microorganisms use complex sensory systems to control their movements in response to stimuli, which allows them to migrate to optimal environments [51]. Of note, most of these surface motility mechanisms have never been studied in microgravity conditions.

2.2 Swimming in the flow

Microorganism swimming behaviour is possible through the flagella-driven motion. The structure and functioning of flagella are different between eukaryotic and prokaryotic cells. The prokaryotic flagellum on which we will focus in this review acts as a reversible rotary motor powered by transmembrane proton potential (a different proton concentration is a priority for its function). It is composed of an anchoring basal body, a hook and a long helical filament [52]. The anchoring basal body acts as the rotary motor of the structure, the hook is the junction structure that connects the motor and filament, and the flagellar filament is normally a left-handed helix of a length of 5 to 10 µm and a diameter of 20 nm. When motor rotation is counterclockwise (CCW) the cell body is pushed and starts its motion in a linear trajectory at an average speed that is for example about 40 µm/s in the case of *E. coli*. When motors rotate clockwise (CW), the filaments are placed under right-handed torsional stress, resulting in a filament poorly defined orientation resulting in tumbles and a phase of random reorientation. This type of behaviour can be mathematically characterized in an isotropic environment using the Persistence Random Walk model (PRW) described by Dickinson & Tranquillo [53]. In this model cells trajectories are described by a succession of uncorrelated movements of a characteristic duration (the times between two different tumbles). Motility is quantified by three parameters: root-mean-squared speed, directional persistence time, and random motility coefficient (analogous to a molecular diffusion coefficient) [54]. The random motility of microorganisms is lost in the case of an anisotropic environment where cells sense chemical and physical gradients resulting

in directional motility (taxes). These directional motions are categorized based on the stimuli depending on chemical (chemotaxis [55], aerotaxis, [56]), thermal (thermotaxis [57]), electromagnetic (magnetotaxis [58]), and light intensity (phototaxis [59]) special gradients. In an anisotropic environment, random reorientation after a tumble also occurs, but the different duration of motion phases is observed among different directions of motion with respect to the direction gradient. A motion toward the gradient persists for a longer time with respect to the case of motion in the opposite direction [60].

Another source of environmental anisotropy can be induced by mechanical stresses, that can be related to flow directionality or force fields, such as gravity. In particular hydrodynamic shear plays a key role in biofilm formation and morphology [61]. In recent work, Rusconi et al [62] demonstrated that shear flow produces spatial heterogeneity in bacterial distribution inside a microfluidic channel. Shear flow seems to affect bacteria accumulation at the channel wall boundary, in a so-called "trapping effect". This effect is a function of the shear rate in a given range of shear. This flow effect hampers chemotaxis and promotes surface attachment. These results prove that flow influence can overcome taxes and directly affect the first step of biofilm formation (adhesion on surfaces). In other recent works, the effect of flow was evaluated not only for its contribution to cell swimming behaviour but also for its effect on biofilm morphologies [62] [63]. A common observation can be made from these studies: at low shears, biofilms present a lower cohesion resulting in loose top layers. Recent studies show that high shear causes a faster diffusion of nutrients and higher incorporation of bacteria, promoting the formation of more crosslinks in the EPS matrix and, ultimately, a more mechanically stable biofilm [64].

In space-relevant applications, specific conditions, such as microgravity, can impact swimming motility, and bacterial growth, but the research available so far seems to be controversial on this aspect. In the review of Benoit and Klaus [65], it is found that spaceflight and devices simulating microgravity enhanced non-motile microbial growth in a liquid medium. A common explanation of this phenomenon is related to two gravity-related effects: the sedimentation of cells, and the potential buoyant convection of less dense fluid in the proximity of the cell. In microgravity conditions, both

these phenomena are reduced and as a result, bacterial cells are more uniformly distributed in the liquid medium, in an environment governed by Brownian diffusion. Motile swimming bacteria seem to reduce this phenomenon, by actively agitating the surrounding quiescent fluid with flagella rotation and reducing the difference between 1g gravity and microgravity condition. In contrast with this hypothesis, a recent study [66] observed three different strains (non-motile *Sphingomonas desiccabilis CP1D* and motile *Bacillus subtilis NCIB 3610*, *Cupriavidus metallidurans CH34*) exhibiting the same cell final concentration after 21 days in space growth, respect to standard ground controls. This controversy suggests that microgravity effects on bacterial growth and the role of cell motility related to this aspect are still not well understood, and deserve further investigation. The definition of a standard protocol to compare bacterial growth and biofilm formation in different gravity conditions is also still not defined.

Bacterial motility deeply affects the colonization of surfaces both in no-flow and flow conditions, due to the forces generated by the flagellar-fluid motion at the microscale and the elongation of the cell body. In no-flow conditions, the surface accumulation of motile bacteria is promoted by the hydrodynamic interaction between the swimming cell with the solid surface [67]. This phenomenon, combined with stop events and transient surface adhesions, allows bacteria to attain optimal surface diffusivity [68]. In flow conditions, hydrodynamic interactions trigger bacterial motion in the direction opposite to the flow, leading to upstream flagellar swimming [69]. Upstream motility can also be achieved by surface motility with type IV pili, as shown in *P. aeruginosa* [70] and *Mycoplasma mobile* [71], with a lower velocity compared to upstream swimming. In both cases, the torque exerted by flow shear rotates the cells around the appendages-free extremity of the body and orients them facing upstream, resulting in a preferential direction of motion. Upstream migration grants an advantage in the colonization of flow networks [72] and promotes the segregation of bacterial species based on their surface motility [73]. Due to its significant implications for bacterial spreading on surfaces, upstream migration should be accounted for while evaluating the origin of bacterial contamination in technological settings.

2.3 Moving as a free cell or a group on the surface

The multiple strategies employed by bacteria to move on surfaces (swarming, twitching, gliding, sliding) are important for survival since they govern the dispersal of progenies, and the way bacteria aggregate into microcolonies under unfavourable conditions, typically starvation or oxygen depletion. Twitching motility is a key mechanism for many pathogenic strains to propagate on surfaces, either as individual cells or collectively. This type of motility is found in many biofilm-forming species, such as *Pseudomonas aeruginosa*, *Neisseria gonorrhea*, *Myxococcus xanthus* or *Acinetobacter baumannii* [74] [75]. Twitching allows single cells to move on surfaces at typical speeds of the order of a fraction of a micrometre per minute and is powered by type-IV-pili (T4P). T4P are thin (~10 nm) contractile surface appendages, up to several micrometres-long and often polarly localized, with a terminal adhesin that can act like a hook and promiscuously bind surfaces. T4P are formed by the assembly of a protein subunit: polymerization/depolymerization cycles at the base of the appendage power motility, and pili extension-attachment-contraction-detachment cycles propel bacteria through surfaces [76] [77][78]. By allowing bacteria to explore surfaces and efficiently colonize different microenvironments, twitching motility is one of the key strategies allowing bacterial dispersal, pathogenesis and is also an important ingredient of biofilm development [79] [80] [81]. In flow conditions, the polar localization of pili results in the upstream migration of adhered bacteria, a counter-intuitive effect that can provide a dispersal advantage for twitching species [70][73]. Recently, it has been shown that mechanical signals sensed and transmitted by T4P regulate virulence factors in *P. aeruginosa* [82] or direct twitching motility of individual bacteria [83], and suggested that substrate rigidity could modulate bacterial twitching, thus impacting colony morphogenesis [84]. Together these findings highlight the importance of mechanical interactions between T4P and their environment, which could be modified in space conditions.

However, so far it is unclear whether pili-mediated motility is modified under microgravity conditions, and no experimental nor theoretical study has focused on this specific point.

One basic question is to determine whether the direction of twitching bacterial displacement steps could be biased by gravity, and thus modified under zero-g conditions. If evaluating the forces at

stake, it appears very unlikely that gravity could impact the behaviour of a single twitching bacterial cell: T4P stall forces, which drive bacterial twitching on surfaces, have been measured to range from 50 to over 100 pN. In contrast, the gravitational force on a cell (in air) of volume 3 x 2 x 2 μm^3 and density ρ=1 g/ml is ρVg=0.1 pN and could therefore play no role in the twitching process. Following this reasoning, only cohesive colonies of a few dozen bacteria could be exposed to gravitational forces comparable to T4P contractile forces.

However, it is entirely possible that microgravity conditions could modify pili expression levels or activity, thus impacting twitching motility. This is supported by several studies that showed that signalling pathways are modified under microgravity [85].

A modified twitching behaviour could have important consequences: twitching impacts surface colonization, and more generally the early spatial organization of bacteria into colonies, which can directly impact their tolerance to environmental stresses in general, and in particular to the action of antimicrobials [86]. Second, by translocating across substrates bacteria can actively modify the underlying surface by depositing extracellular polymeric substances [87]. This coupling between environmental conditions (shear flow, substrate mechanical and chemical properties), surface motility and EPS distribution governs microcolony formation and should thus be considered when designing space equipment.

Surface motility and dispersal of individual bacteria can also take place through mechanisms that do not require any active surface appendage: gliding or sliding.

Gliding is common among Myxobacteria and is also observed in a number of phylogenetically diverse gram-negative, non-flagellated bacteria [88]. It relies on the movement of adhesion complexes along helical tracks on the cell surface, powered by proton-activated molecular motors [89]. Gliding propels the cell body forward at several micrometres per minute. It is known that gliding cells deposit a layer of slime on the substrate, and the role of this thin slime layer as a lubricant for cell displacement is well-established. In addition, the trafficking of adhesion complexes on the helical MreB scaffold results in sinusoidal deformations of the cell surface. Recently, Tchoufag et al. proposed that these periodic deformations, when transmitted to the underlying elastic slime layer, result in local pressure gradients that generate the overall thrust force experienced by bacteria [90]. Interestingly, their

elastohydrodynamic model accounts for the known substrate-rigidity dependence of gliding motility, which decreases on soft substrates, similar to twitching [84]. Stall forces of gliding molecular motors were measured around 12 pN, resulting in total gliding forces up to 60 pN (with ~ 5 motors/bacteria) [91].

In conclusion, the forces exerted by individual gliding bacteria are similar to the ones involved in twitching motility, and thus the physical mechanisms that govern these processes are unlikely to be directly influenced by gravity. However, indirect regulation of these phenomena could exist in space conditions, as a result of changes in bacterial phenotypes (e.g. T4P or EPS expression levels).

Once they form a microcolony, bacteria can collectively slide on surfaces thanks to division and growth: dividing bacteria at the centre of a colony generate pressure, pushing their neighbours outwards. The progression of the edge of the colony can be facilitated by the production of biosurfactants that reduce friction [92] [93], EPS that trigger osmotic swelling of the biofilm [94] or capillary forces at the air/liquid interface [95]. This gives rise to very diverse colony morphologies, including fingering instabilities [96]. To our knowledge, this phenomenon has not been specifically studied under microgravity or 0g conditions. Because bacteria adhere to each other and to the underlying substrate, growth gives rise to local stress build-up in the colony, which relaxes through rapid reorganization events. Maximal adhesion forces in these “focal adhesions” under spreading colonies have been measured experimentally of the order of 50 to 100 pN [97] -again in the same range as the forces involved in twitching or gliding motilities.

Could the presence of gravity directly impact macroscopic biofilm spreading? The capillary length $\sqrt{(\gamma/(\Delta\rho g))}$ of a water droplet under 1g conditions is ~3mm, meaning that gravity would only deform droplets larger than this characteristic size. Considering that biofilms have a density close to water’s, and even if the production of biosurfactants decreased surface tension, the capillary length would not go under a few 100 µm. This means that only thick, mature biofilms could potentially be deformed by gravity under their own weight. The structural differences observed for *P. aeruginosa* biofilms in spaceflight conditions [] are most likely due to nutrient or oxygen availability, or changes in the motility of bacteria, rather than the absence of a direct deformation of biofilms by gravitational forces.

In specific conditions, billions of bacteria can migrate cooperatively from a colony across distances of centimetres in a matter of a few hours through a phenomenon called swarming [98]. Swarming motility is a process by which bacteria can rapidly advance on moist surfaces in a coordinated manner [99]. It is a multicellular, flagella-mediated surface migration of bacterial groups typically involving surfactant secretion and an increase in flagella numbers [100] [98]. In *Bacillus subtilis*, this developmental process is observed on semi-solid agar (0.6%–1% agar) and has been shown to be completely dependent on flagella and surfactin production [101]. Traditionally, dispersal by microbial swarm propagation has been studied in monoculture, but there is evidence that swarming microorganisms can transport other species by forming multispecies swarms with mutual benefits [102].

2.4 Active microbial movements in biofilm communities, dispersion and hitchhiking

Biofilm structures were initially described as a sessile three-dimensional assemblage of microorganisms immobilized in an EPS organic glue [103]. The combination of new visualisation tools such as confocal laser scanning microscopy (CLSM) along with genetically engineered fluorescent reporter strains allowed the discovery of unexpected mobile subpopulations within biofilms. In the early 2000s, Tim Tolker-Nielson and his collaborators demonstrated in a series of articles on *Pseudomonas aeruginosa* the migration of a subpopulation of cells to the cap of mushroom-like biofilm structures [104]. These movements involved type IV pili and were observed on the interface between the biofilm and the bulk fluid [105]. The biological role of death and lysis in biofilm development and the existence of hollow voids containing cannibal swimming subpopulations of cells involved in active cell dissemination was also pinpointed [106] [107]. Several authors demonstrated that non-flagellated bacteria were also able to actively disperse the biofilm population. This is the case of the coccoid pathogen *S. aureus* for which the induction of the *agr* system in established biofilms detaches cells through a dispersal mechanism requiring extracellular protease

activity [108] [109]. More recently, it was shown that flagella-propelled motile bacilli were able to swim and create transient pores within the biofilm matrix, increasing the macromolecular transfer with the bulk phase [110] [111] [112]. While these bacilli swimmers can deliver locally several types of effectors, it was shown that they can actively transport several types of adsorbed organisms taking advantage of a "free ride" inside the biofilm. Described "hitchhikers" on the flagella of motile bacilli comprise several families of non-motile organisms such as the bacterial pathogen *Staphylococcus aureus* [113], fungal spores [114] and bacteriophages [115] [116].

3. Emerging properties of surface-associated migrating communities

Understanding how microorganisms adapt to stressful space conditions has been the focus of many studies. Most studies involved single bacterial species, often pathogenic, including various Pseudomonas, Enterobacteria such as *E. coli* and Salmonella, Actinobacteria, and bacteria of the Streptococcus and Enterococcus genera [117] [118] [119] [120] [121] [122] [30] [123] [124] [125]. Non-pathogenic species such as the soil bacteria *Bacillus subtilis*, the fermentative bacteria *Lactococcus lactis* or the nitrogen-fixating bacteria *Rhodospirillum rubrum* have been also sent to space [126] [127] [128] [129] [130]. Experiments conducted on spaceflight as well as on ground-based simulators established that microgravity triggered various physiological responses by affecting bacterial cell growth, cell morphology, gene expression, gene transfer, virulence, drug resistance, biofilm formation, and secondary metabolism [117] [121] [130] [125] [131].

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3.1 Impact of spaceflight conditions on the adaptation of bacterial populations

Phenotypic changes with potential implications for astronauts have been reported in various bacterial species exposed to low-sheared modelled microgravity (LSMM). Growth under simulated microgravity conditions increased the cell density of *Stenotrophomonas maltophilia*, *Lactobacillus acidophilus* and *Pseudomonas aeruginosa* [30] [130] [132]. Notably, several studies conducted under space flight conditions have linked growth rate to bacteria motility, suggesting that the effect of microgravity could be indirectly caused by a lack of convective flows altering the diffusional access to nutrient and affecting the immediate cellular metabolic environment [30] [131]. A direct consequence of this hypothesis is that this response should be counteracted by motility, as corroborated by a comparative study between a wild type and a Δ*mot*ABCD motility-deficient mutant of *P. aeruginosa* exposed to a space flight environment [30] (Kim et al., 2013b). In *Streptococcus mutans*, it was shown that genes involved in carbohydrate metabolism, translation or stress responses were differentially expressed in simulated microgravity conditions, with potential effects on the cariogenic potential of this bacterial species [133]. Phenotypic changes were also observed in *E. coli* when cultured in space, along with an increase in cell size, cell counts, and cell envelope thickness. Compared to earth, *E. coli* cells challenged to microgravity also exhibited higher resistance to gentamicin sulfate coupled with a unique ability to generate numerous outer membrane vesicles (OMG), these two phenotypes being connected to a change in membrane fluidity [134] [125]. Long-term exposure to microgravity indeed affects bacterial virulence as well as susceptibility to diverse antibiotics and drugs in many bacterial species. Increased virulence has been observed in bacteria pathogens grown in simulated microgravity and space conditions [135] [136] [137]. After exposure to simulated microgravity in rotating-wall vessel bioreactors, the pathogen *Salmonella typhimurium* became more virulent in mouse or cellular infection models [138]. In their study, Gilbert and al. revealed that the opportunistic pathogen *Serratia marcescens* was more lethal to *Drosophilia*

melanogaster after exposure to true spaceflight conditions [135]. Importantly, they also established that this characteristic did not persist after the cells resumed normal growth under ground conditions. This observation suggests that microgravity can induce transient physiological changes in microorganisms.

Another major concern is that prolonged exposure to microgravity conditions triggers increased antibiotic resistance, as documented for *E. coli, S. aureus, Streptococcus pyogenes, P. aeruginosa,* or *Enterococcus faecalis* [139] [140] [137] [141] [125] [142]. It was proposed that adaptive resistance to antibiotics under low gravity in *S. aureus* and in *E. coli* could be associated with modifications of the cell envelope such as an increase in membrane fluidity and cell wall thickness [143] [144] [125]. Short-term microgravity (<50h) also demonstrated the potential to affect *E. coli* resistance to antibiotics from different families including gentamicin, ampicillin, nalidixic acid, penicillin G or chloramphenicol [145] [146] [147].

In addition to phenotypic changes, the question of the genetic evolution of bacterial populations under spaceflight and microgravity conditions and its role in the emergence of particular bacterial phenotypes, such as resistance to antimicrobials is particularly of concern in a spacecraft environment during long-term missions. Interestingly, mutation frequency and/or spectrum of mutations in the *rpoB* gene involved in rifampicin resistance was modified in *Staphylococcus epidermidis* and *Bacillus subtilis* cultures grown in spaceflight environments (ISS) by comparison to ground control cultures [148] [149]. That supports the idea that space environments can induce unique stresses on bacteria, leading to modulations in their mutagenic potential. Through a pangenomics meta-analysis of 189 genomes of *Bacillus cereus* and *Staphylococcus aureus* from different origins, Blaustein et al. (2021) [150] identified genomic signatures specific to International Space Station (ISS) bacteria. Functions related to biosynthesis, materials transport, or stress response were enriched in ISS-derived strains suggesting their involvement in bacterial survival under ISS selective pressures.

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3.2 The importance of biofilm lifestyle in adaptive migration of bacterial populations

Biofilms are dynamic multicellular edifices and are recognized as a collective strategy for microorganisms to adapt and survive face changing environmental conditions [31]. It is now acknowledged that exposure to the space environment enhances biofilm biomass and thickness in most bacteria [120] [30] [151] [140] [132] [152] [131] [125]. The increased propensity to develop biofilms in space has been first discovered in *P. aeruginosa* [151]. The typical column-and-canopy-like architecture revealed during the space shuttle Atlantis missions illustrated the complex selective forces at play that shaped the 3D structure of biofilms when exposed to microgravity [153] [151]. The formation of such structures requires flagella-driven motility but is not dependent on the carbon source [154]. Alteration of biofilm mass, composition, and architecture, combined with abnormal EPS distribution has been also reported for *Streptococcus mutans* grown under simulated microgravity [155]. Substantial modifications of biofilm architecture and colony morphology, associated with an increase in virulence and resistance to environmental stress and antifungal (amphotericin B), were also observed for *Candida albicans*, an opportunistic fungal pathogen, grown in low-shear modelled microgravity bioreactors [156] [157]. To illustrate the effect of microgravity on biofilm cell metabolism we simulated the growth of microalgae biofilms in both terrestrial and microgravity conditions (**Figure 4**). These simulations suggest that micro-gravity impacts the spatial structure of the biofilm and therefore the resulting substrate consumption and the overall biofilm growth.

One key component of the survival strategy of the biofilm community is the ability to withstand externally applied mechanical stresses, thanks to the viscoelastic nature of the EPS matrix. When a force is applied, biofilms instantaneously undergo an elastic deformation as solids and then slowly flow as viscous fluids, further spreading on surfaces while maintaining their structural integrity [155] [156]. The viscoelastic behaviour increases the surface spreading [157] and allows the formation of biofilm filaments suspended in the bulk fluid, known as biofilm streamers [159]. The EPS matrix

supports the mechanical stability of the biofilm through physicochemical interactions, and EPS biochemical composition determines its mechanical behaviour [159] [160]. Biofilm mechanical behaviour is key to the impact of biofilms in technological contexts, including spaceflights. However, while the impact of microgravity on biofilm architecture and composition has been elucidated, the microgravity-induced changes in biofilm's mechanical behaviour are still understudied. Measuring the ability of biofilms to withstand stress would provide information and indicates future directions for the design of biofilm-cleaning tools. Additionally, the mechanical protective role of the matrix is largely decoupled from the viability of the cells themselves, so even after successful antimicrobial treatment, the detrimental effects of biofilms due to fouling persist beyond the death of the cells.

In multispecies communities, a consequence of microgravity-induced modification of biofilm structure is a modification of competitive interactions, resulting in a modification of ecological balance and an alteration of community functions. This was illustrated by the fitness increase of *S. mutants* over *S. sanguinis* when mixed under simulated microgravity compared to ground level [161] which would promote the initiation of dental caries in dental biofilms. Similarly, by performing a shotgun metagenomics analysis of ISS environmental surfaces, Singh et al. [162] demonstrated a specific composition of ISS microbial communities compared to earth analogous. Moreover, the authors reported an increase in antimicrobial resistance and virulence gene factors over the period sampled showing the specific adaptation of functional profiles of ISS microbial surface-associated populations [162]. Overall, these observations emphasize the close interplay between the three-dimensional organization of biofilm, its plasticity and the modulation of functional properties in response to microgravity conditions.

Furthermore, biofilm structural changes in spaceflight environments are likely to affect how bacteria evolve toward specific genotypes. Biofilms are considered incubators for microbial genetic diversity as they promote the process of diversity generation and protect genetic diversity [163]. This mainly arises from the multiple micro-environments produced by the chemical gradients and the protective three-dimensional structure. This phenomenon plays a central role in microbial adaptation and in the "migration" toward specific functions expressed at the scale of the whole community such as

antimicrobial resistance. This feature, in combination with the fact that spaceflight conditions can independently affect mutagenic potential in bacteria [148] [149] underlines the need to better understand the adaptation of surface-associated migrating communities to spaceflight environments.

More generally, biofilms represent a spatial and structural advantage for cell-to-cell communication through both metabolic and genetic exchanges. Indeed, in bacterial populations, the emergence of functional traits is much associated with the horizontal transfer of genetic determinants. Considering antimicrobial resistance, the emergence of resistance at the community scale relies on their propensity to exchange plasmids, transposons and other genetic determinants considered reservoirs for antibiotics genes. In a recent study, Urnaniack et al. established that microgravity stimulated the horizontal transfer of two antibiotic resistance genes, *blaOXA-500* and *isaba1*, from *Acinetobacter pittii*, in four *S. aureus* strains, thus posing the hypothesis that interspecies genetic transfer could also occur onboard of a space station [141]. This study points to the potential role of other modes of genetic transfer such as natural competence and phage transduction in spreading resistance genes and pathogenicity determinants in space. The facilitation of horizontal gene transfer in biofilms is proposed to be part of the mechanisms responsible for the dissemination of virulence and antibiotic-resistance genes in space [164] [137].

The microbial stress response to microgravity is thus multifaceted. Understanding how microbes integrate information from a microgravity environment to elicit multiple and interconnected phenotypes requires understanding at a system level. Although the effect of microgravity in biofilm formation is well documented in the literature, knowledge remains to be gained to understand the decision-making genetic circuits underlying this lifestyle switch.

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3.3 The effectors and what we know about their expression in space conditions

Deciphering the principles underlying the cellular response to altered gravity is expected to provide important information for the development of countermeasures to control bacteria growth, virulence, antimicrobial resistance and biofilm formation in space. Whole-genome gene expression profiling offers the prospect of gaining insight into gene regulatory pathways and elucidates the effectors involved in adaptation to microgravity. In the last decade, advances in omics approaches have enabled the generation of data to identify potential microgravity-sensitive genes. Several studies made under real or simulated microgravity environments provided the differential-gene expression analysis of bacterial genomes, which partly supported the observed phenotypic changes [120] [165]. Transcriptomic analysis in various bacteria exposed to altered gravity highlighted the differential expression of genes involved in motility and transport, including multidrug efflux systems and metal-ion transport and utilization. Confusingly, different *E. coli* strains exhibited either an increase or a decrease in the expression of flagellar and motility-associated genes as well as chemotaxis-associated genes under simulated microgravity [166] [167] [168]. These different responses were reflecting their distinctive motility capabilities, their physiological stages in the experiment, as well as the different nutrient composition of the medium tested [166] [167] [168]. In a different approach, whole-genome sequencing of *E. coli* cells exposed to LSMMG microgravity for up to 1000 generations revealed loss-of-function mutations affecting genes of the flagellar, motility and chemotaxis regulons [169] [170]. Genes encoding proteins that compose the flagella apparatus were reproductively down-regulated in the pathogen *Salmonella typhimurium* when exposed to spaceflight or to simulated microgravity conditions [171] [172]. Real and simulated microgravity commonly elicited the differential expression of chemotaxis genes in the gram-negative pathogen *P. aeruginosa* [119] [173]. All these studies underlined the importance of motility and chemotaxis in bacterial adaptation to microgravity conditions. Lately, Su et al., used an integrated multi-omic approach combining transcriptomic and proteomic to investigate the impact of long-term exposure to microgravity on *Stenotrophomonas*

maltophilia physiology and metabolic responses [132]. Gene ontology enrichment analysis revealed that simulated microgravity conditions affect several processes related to cell adhesion, motility and biofilm formation. Most particularly, genes encoding proteins that compose the T4P pilus machinery and two-component systems (TCSs) are up-regulated, in keeping with the physiological changes observed under microgravity such as enhanced biofilm formation ability, and increase growth rates [132].

Transcriptomic study in the gram-positive bacteria *Streptococcus pneumonia* revealed that exposure to microgravity conditions up-regulated many genes involved in the cell envelope biogenesis, DNA replication, recombination and repair as well as ABC-type multidrug transport systems [120]. However, a unique comparative transcriptomic analysis from a *B. subtilis* strain grown under identical conditions aboard ISS in two separate spaceflight experiments BRIC-21 and BRIC-23, provided invaluable data on the bacterial response elicited under microgravity [165]. This study revealed higher levels of transcripts related to anaerobic respiration, the production of secondary metabolites (e.g; siderophores), the synthesis of antimicrobials (e.g. bacteriocins), as well as the utilization of various nutrients [165] (**figure 5a**). These observations correlated with the limitation in oxygen and nutrient transport due to the lack of convection in the absence of gravity, as mentioned above. However, one of the most interesting outcomes of this comparative study was the overexpression of genes involved in biofilm and motility pathways [165]. Although the domesticated *B. subtilis* strain 168 used in these experiments was not prone to form strong biofilms, clusters of biofilm-related genes were significantly upregulated in the two experiments, such as parts of the *epsA-O* operon, encoding the exopolysaccharide production machinery, and genes of the *tapA-sipW-tasA* operon encoding important components of the biofilm matrix (**figure 5a**). Another regulatory function related to the biofilm lifestyle switch is also highlighted by the increased expression of the *sivA*, *B* and *C* genes encoding factors that modulate the activation of the sporulation master regulator Spo0A [174]. Notably, *sivB* encodes the BslA protein, another component of the extracellular matrix of the *B. subtilis* biofilm [175]. Finally, illustrating another form of motility behaviour, the up-regulation of the *srfAA-srfAD* operon, involved in the production of surfactin, suggested an increased ability of Bacillus

to swarm across solid or semi-solid surfaces under microgravity. An effect of microgravity on swarming motility was also strengthened by the up-regulation of the entire *yrkEFHIJ* operon, encoding genes of unknown function but found to be specifically expressed during swarming conditions [176] [177].

3.4 The role of global regulators in the adaptive response to space conditions

Global RNA-seq based gene expression and proteomic analysis uncovered hundreds of genes differentially expressed under low gravity compared to earth gravity conditions, part of them being involved in cellular pathways governing the observed physiological responses. Their role in bacterial adaptation to microgravity might be direct or indirect. Bacteria adapt to a stressful environment by reprogramming gene expression to produce the necessary effectors to cope with stress. Responses involved key regulators that allow the rapid modulation of the expression of a wide range of genes. In experiments on rotating HARV (high aspect ratio vessel bioreactors) in Salmonella, the LSMMG regulon comprises 163 genes, involved in various cellular processes, part of them known to belong to other specific regulons governed by global regulators [172]. The analysis identified two chromatin-associated proteins, HimA and DPS, able to affect local gene expression by modulating DNA topology [178]. Among other transcriptional factors, the ferric uptake regulator Fur is also involved in the LSMMG response [172]. In *P. aeruginosa*, the alternative sigma factor AlgU controls genes involved in alginate biosynthesis and oxidative stress [179]. AlgU has been found to play a role in the adaptive response to LSMMG, in agreement with the observed enhanced biofilm and virulence phenotypes [119]. Recently, the global regulator Hfq has emerged as a recurring space-responsive gene in *E. coli*, *S. typhimurium*, *Vibrio fischeri* and *P. aeruginosa* [119] [173] [180] [29]. Hfq is a RNA-binding protein acting as a global post-transcriptional regulator of gene expression in bacteria. Hfq acts as a RNA-chaperone, stabilizing RNA-RNA interactions, such as those occurring between

small regulatory RNAs (sRNAs) and their messenger RNA (mRNA) targets, thus modulating their function by multiple mechanisms. Owing to its functional flexibility, Hfq participates in regulating various bacterial processes, including motility, biofilm formation and virulence [181] [29] [182]. Hfq expression was found consistently down-regulated in several space transcriptional studies [183] [119] [173] [180] [184] [29] [172]. Considering that most of the flagellar genes are regulated through Hfq-dependent sRNA in many Gram-negative pathogens, Hfq is thus arising as a central player in motility behaviour under microgravity stress across gram-negative bacterial species [183] [173]. Contrastingly, Hfq was not identified in the transcriptomic profiling of *B. subtilis* in space conditions [165]. In this Gram-positive bacteria, the Hfq protein is not essential although it plays an important role in survival during the stationary growth phase [185] [186]. Compared to Gram-negative bacteria, Hfq in *B. subtilis* Hfq does not play a central role in post-transcriptional regulation and its absence alters the expression levels of only a limited number of genes. Most particularly, genes involved in the anaerobic respiration and fermentation pathways and belonging to the ResD/Rex regulons, are up-regulated in a Δ*hfq* mutant [185]. Interestingly, the comparative analysis of the *B. subtilis* transcriptomes from the BRIC-21 and BRIC-23 spaceflight missions revealed a down-regulation of expression of many operons regulated by ResD (6 over 17) and Rex (5 over7) (**figure 5b**). This observation suggests a less direct role of the Hfq-mediated response to space conditions. Further studies are required to decipher the genetic regulatory network at play during the adaptation of a bacillus cell to microgravity stress.

4. Controlling microbial migrating communities on spaceflight habitats surfaces

In an event of a spaceflight biocontamination outbreak, such as the fungal contamination of panel fronts in the “hygiene area” of a functional cargo module or clogged lines in SRV-K line of the

condensate recovery system (**Figure 1**), remediation actually relies on cleaning and disinfection with fungistat wipes, air filtration with POTOK 150MK system, or disassembly and replacement of contaminated payload [17]. However, these strategies are not feasible for extended long-term human missions to space and special concern is raised about microbial biofilms because of their difficulty to be eliminated due to their increased resistance to antimicrobials. Indeed, biofilms are the most resilient form on life on Earth and currently available coatings and antibiofilm technologies, are not yet able to permanently avoid biofilm growth. This limitation is relevant both on Space and Earth applications. A possible approach is to adopt combined strategies to delay as long as possible biofilm formation (coatings, biocides, shear stresses…). In a recent review, H.-C. Flemming concluded that to really solve biofouling problems, it is necessary to learn how to live with biofilms and mitigate their detrimental effects instead of trying to eradicate them [188]. Hence, new strategies are being investigated to prevent microbial migrating communities on surfaces in order to reduce microbial risk to crew health, safety, and performance during human exploration in space. In this regard, Zea et al. [3] summarized potential biofilm control strategies for extended human spaceflight missions including, biocides, coatings, ionizing radiation, biofilm detachment, biocontrol as well as chemical removal of nutrients. It was pointed out that solutions developed against biofouling of marine surfaces and medical devices could bring insights useful for biofilm control on spacecraft. The aim is to develop broad-spectrum antibiofilm surface treatments for confined space stations which would be easy to upscale. Representative coatings for biocontamination control are typically based on metal ions (silver(I), copper(II), tributyltin), titanium alloys and mixtures, synthetic polymers (e.g. polyethylene glycol PEG) that can be copolymerised with hydrophobic polydimethylsiloxane (PDMS) or biopolymers such as poly(3-hydroxybuyrate-co-3-hydroxyvalerate) [189] [190] (**Table 1**). The active molecules can be deposited or chemically grafted on the surface. It can also be formulated to be released progressively, locally or on-demand [191]. Special emphasis is put on the lack of toxicity and long-term stability under space station conditions. Wang et al [192] reported the strict standards that antimicrobial coatings must-have for space application, according to the European Cooperation for Space Standardization (ECSS). Forbidden powder (e.g. Cd, Zn, Hg, polyvinyl chloride and radioactive materials (European Cooperation for Space Standardization, https://ecss.nl) and key

parameters such as toxicity, flammability, stability, effectiveness for the application, maturity of use, program/user acceptability, and material compatibility are taken in the exam. A strong limitation in the validation of these antimicrobial surfaces is the distance between laboratory experiments (short-time scale, monospecies contamination in rich synthetic media…) and environments existing in the real world [188]. Moeller and collaborators designed the ISS experiment "BIOFILMS" [193] [25] tol investigate the formation of biofilms on various antimicrobial surfaces in a real space station environment. These materials include inert surfaces such as stainless steel, as well as antimicrobial active surfaces such as copper-formulated materials. Antimicrobial compounds involved in those formulations raised major concerns as their continuous use may lead to the emergence of antimicrobial resistance (AMR) [194]. In this context, coatings free of heavy metals are now under investigation in the framework of ESA's NBactSpace project implemented by the Luxembourg Institute of Science and Technology [192].

A promising, biomimetic, method for biofilm prevention is based on the example of several natural super-repellent surfaces [195] which exhibit non-sticking properties by combining hierarchical micro/nano-structures with low surface energy agents. The most prominent example is the surface of the lotus (*Nelumbo nucifera*) leaf which corresponds to a very large water contact angle (>150°) and small sliding angle and contact angle hysteresis (<10°) [196] thus exhibiting both superhydrophobicity and extreme water repellency [197]. Lotus-like phenomena are typically studied in three-phase systems which include air, a liquid and a solid). However, the same concept can be applied on a three-phase system consisting of microbes, a liquid medium and a solid surface. Several methods have been developed to produce lotus leaf-like materials which have the potential to be used as surface coatings for biofilm prevention [198], according to a two-step process [199]: First, a non-sticking surface can resist or prevent the initial attachment of microbes. Second, even if there are some microbes adhered to the surface, these can be easily removed by slight external forces e.g. wiping, wind, and water impact [199]. According to these concepts, superamphiphobic materials, which show extreme non-sticking properties as they have the ability to repel not only water but

729 virtually any liquid, may offer enhanced protection against biofilm formation [200]. It is stressed,
730 however, that non-wetting conditions do not always promote microbe repulsion. For example, Yuan *et*
731 *al.* [201] showed that polystyrene surfaces with a moderate water contact angle of about 90° produced
732 the highest level of bacterial (*E. coli*) adhesion whereas limited bacterial binding was observed on
733 both superhydrophobic and superhydrophilic structured polystyrene surfaces. Moreover, it is well
734 known that superhydrophilic and underwater superoleophobic (SUS) materials have in general
735 antifouling properties as they repel organic materials [202]. Prevention of biofilm formation by
736 antiadhesive surfaces can be supplemented by a biocidal step. This is of particular interest in the case
737 of brush coatings that have been shown to increase antimicrobial action in addition to reducing
738 bacterial adhesion forces with the material [203]. Some specific material also exhibits strong
739 antibacterial activity without being formulated with biocides. Ivanova *et al.* [204] showed that
740 bacteria entering in contact with the array of pillars of the superhydrophobic surface of cicada
741 (*Psaltoda claripennis*) wings are inactivated within a few minutes. Clearly, this bactericidal ability of
742 the cicada wing surface is a physicomechanical effect as it does not involve the action of any biocide
743 [204]. With similar biomimetic nanopatterned surfaces, Michalska et al. demonstrated the dependence
744 upon pillar density and tip geometry on the mechanism of bacterial killing [205]. In a recent study, Pal
745 *et al.* [206] produced a highly hydrophobic laser-induced graphene film that can be implemented on
746 reusable surgical protective masks. Several reports described the combination of superhydrophobic
747 coatings with biocidal agents embedded within the structured coatings [199] including quaternary
748 ammonium compounds [207], metal oxydes [199], N-halamines [208] and natural antibacterial agents
749 [209].

750 Several papers reported the possibility to interfere with microbial processes involved in adhesion,
751 migration and biofilm maturation. Many bacteria produce extracellular adhesins or appendages to
752 mediate their adhesion to the surface. Several coatings containing pilicide molecules such as disperse
753 red 15 or verstatin were found efficient to interfere with the pili function and reduce pathogen fixation
754 [210] [211]. Drugs specifically interfering with flagellar motor assembly and function (agaric acid,
755 phenamil, amiloride) were also reported and could be integrated into such targeted approaches [212]

[213]. A very exciting direction in these applications is to interfere with microorganisms signalling systems and decision-making. Each cell at a specific period integrates hundreds of environmental signals to adopt a specific cell fate. Coatings integrating molecules perturbating the Quorum-sensing (QS) response are already available (Table 1) and [214]. By preventing the biofilm QS-maturation, several important mechanisms of persistence could be bypassed. The cyclic-di-GMP pathway could be similarly targeted to prevent the physiological transition from planktonic to biofilm in many bacterial species. In recent years, several plant metabolites and their formulations (resveramax, cinnamic acid,…) have been identified in motility-swarming-biofilm inhibitors with the advantage of being environmentally friendly and poorly toxic for humans in contact [213].

A microbe-based preventive strategy to protect surfaces from being colonized by unwanted microorganisms is based on guided microbial ecology and interspecies competition. The concept here is to consider that if any surface supports microbial life it is worth settling selected beneficial organisms able to exclude unwanted microorganisms. This positive biofilm approach is applied in a One Health context to limit microbial pathogens on the surface of livestock buildings [215]. This biological approach has shifted from labs to farms in recent years with various commercial products now on the market in several countries paving the way for applications in spatial missions [216].

When preventive approaches failed and a biofilm is formed, the presence of the extracellular cellular matrix, the spatial organisation of the communities and the associated diversification of cell types will generate emergent properties of the community and recalcitrance to the action of most conventional disinfection treatments [4] [32]. It is frequently stated in scientific publications that microorganisms in a biofilm are typically 1000 times more resistant to biocide action than their planktonic counterparts. While the mode of action of disinfectants depends on the type of biocide employed [224], the low efficiency on surface-associated biofilm communities is still not fully understood. It is now evident that biofilm tolerance to disinfectant is intimately related to the three-dimensional structure of the biofilm, heterogeneous within the biostructure and multifactorial, resulting from an accumulation of different mechanisms [4]. To overcome these disinfection limitations, an attractive strategy is to re-sensitive the biofilm population by targeting the matrix. EPS-degrading enzymes can help disrupt the

783 matrix for more effective removal and disperse bacteria in biofilms for more effective killing when
784 combined with antimicrobial agents [225]. Enzyme treatments are mainly used in the medical context
785 to target recalcitrant biofilm infections by undermining the protective role of the matrix and thus
786 increasing the effectiveness of traditional antimicrobial therapies [226]. Exopolysaccharide-degrading
787 enzymes, such as glycoside hydrolases and glucanohydrolases, have been used to degrade a mixed-
788 species *S. aureus* and *P. aeruginosa* biofilm grown in a mouse model of chronic wounds [227] and to
789 prevent the formation of pathogenic oral biofilms [228]. Since eDNA is a broadly conserved EPS
790 component [228], DNases have also proven to be effective in disrupting biofilms [229], both in vivo
791 and in vitro [230]. In particular, human-derived DNase I is exploited to treat pulmonary infections in
792 cystic fibrosis patients [231] [232]. Moreover, enzyme-based biofilm impairment treatments are
793 finding increasing applications in the food [233] [234] and paper [235] industries, potentially opening
794 the doors for their increased use in the technological sector. In particular, since enzymes require
795 specific physicochemical conditions to maximize their efficacy, their use in the spaceflight context
796 would require studies to assess longevity and effectiveness in the application conditions. It was also
797 demonstrated that tunneling the biofilm matrix by selected bacilli swimmers could resensitize bacteria
798 to the action of biocides by creating a transient vascularization network [110]. Matrix destabilisation
799 is also possible by magnetic disturbance. In a recent paper, magnetic iron oxide nanoparticles were
800 successfully used to disrupt a recalcitrant biofilm upon exposure to a controlled magnetic field [236].

801 Among promising strategies to cope with biofilm development, the use of bacteriophages regained
802 consideration in the last decade. By exploiting their ability to kill their bacterial host, phages have
803 been successfully applied to eradicate biofilm from within [237]. Phage treatments can be based on
804 the use of the whole phage particles, but also on phage-derived antibacterial activities. The main
805 phage-encoded bactericidal enzymes are the depolymerases, lyases and hydrolases, externally
806 associated with the virion tail and able to degrade EPS [238]. Phages also encode lysins acting from
807 inside the bacterial cell and are responsible for cell wall degradation [239]. Similarly to DNase
808 treatments targeting extracellular DNA, endolysins and depolymerases-based treatments have been
809 used to overcome the biofilm matrix barrier. The biocidal potential of these phage-encoded enzymes

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has been demonstrated to prevent the biofilm formation of pathogens in vitro as well as in vivo [240]. The use of integral lytic bacteriophages for bacterial biofilm control has been proven a safe alternative approach to antibiotics and chemical biocides [241]. Although most studies assessing the ability of bacteriophages to reduce biofilm biomass are performed in laboratory conditions, the successful application of phages and phage cocktails has been reported in several medical cases as a last alternative to combat drug-resistant bacterial infections [242] [243]. The narrow-host range specificity of phages combined with the ability of bacteria to rapidly develop defense mechanisms to survive infection could be considered as a limitation of this approach. However, the use of phage cocktails with a broader spectrum of infection has already proved to be very efficient in complement to antibacterial treatments in combating pathogen biofilms within medical devices [244]. We can anticipate that similar strategies could be also used successfully in a spacecraft environment. Furthermore, phages are not motile and the structure as well as the composition of the biofilm matrix acting as a diffusion barrier interfere with their penetration and dispersal within biofilm [245]. The recent discovery that phage could be passively transported by motile carrier bacteria sheds new light on the importance of the role of non-host bacteria–phage interactions on biofilm dynamics [115][116]. This behavior, called “hitchhiking”, opens new avenues to improve phage delivery within biofilms. In addition, a growing number of studies highlight the synergistic action of phages combined with other antimicrobials for the effective eradication of biofilms [246] [247] [248]. Emergent approaches are now combining phages and/or phage-derived products with other nano weapons or bactericidal agents to combat biofilms on earth. Phage-based biocontrol could be used in support of other biofilm eradication strategies to delay corrosion and biofouling in space and more generally mitigate biofilm formation on future missions (ICES-2019-271). How the lack of gravity could influence their diffusion and their interaction with their host is currently under investigation (www.issnationallab.org/iss360/phageevolution-rhodium-scientific-studying-viruses-microgravity/). The efficiency of similar treatment applied to biofilm control in the context of spaceflight remains to be studied.

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The chemical, physical and biological toolbox to control biofilms migrating communities is constantly increasing with innovative prevention and curative strategies. However, their efficacy is not universal and synergetic combined approaches will be needed to prevent biofilm deleterious effects during long-term spatial missions [249].

5. Conclusions & perspectives

The vibrant microbiota migrating and settling on surfaces of spaceflight habitats could jeopardize long-term spatial missions by altering surface and equipment functions and threaten astronauts' health. The most conventional hygienic procedure to control surface-associated microbiota on earth is cleaning and disinfection with highly reactive chemicals [4]. This approach is hardly compatible at large scale with long-term space missions in terms of the quantity of water needed, the absence of drainage in microgravity conditions, the cost of transport of the biocides as well as their potential corrosive, toxic and explosive properties. Space agencies intensify their effort in preventive strategies mostly relying on hygienic design and maintenance and the use of anti-biofilm material in a sensitive part of the spaceflight (e.g. WRS). Most of the activity of those materials and coatings relies on antiadhesive or antimicrobial properties [192]. With our better understanding of the specific physiology of microorganisms living in a biofilm in microgravity conditions, we could envision activating those materials with effectors targeting molecular determinants of biofilm initiation/stability/dispersion (pili, flagella, EPS…) or the regulations pathways involved in the shift between planktonic and biofilm cell fate (cyclic di-GMP pathway, Quorum-sensing signaling…). Physical decontamination procedures based on the intensive exposition of surface to antimicrobials beam (UV, blue light, pulsed light, plasma) are also interesting alternatives to chemical disinfection [250]. Another family of control strategies are based on biological organisms (biological warfare). In several areas, microorganisms are used to kill specific unwanted organisms or to guide the ecology of the surface (microbiota editing). This is the case of phages which are viruses killing specifically a

group of (pathogenic) bacteria or of positive biofilm that is composed of bacteria recognized as safe that are settled on purpose on a surface to prevent unwanted colonisation. Both these microbe-based strategies are already in use in the biomedical, agricultural and food industries. A point of interest is that they can be propagated indefinitely very easily in the space habitat.

The first demonstration of DNA sequencing in space was performed recently by NASA with the portable MinION device (Oxford Nanopore Technologies) on the ISS. Successful sequencing of mouse microbiota (bacterial and viral DNA) was demonstrated, showing potential for monitoring of microbes in food, water and environment [14] [251]. This opens doors to on-site analysis and monitoring of the biofilm species composition and ecological diversity evolution, but also in terms of functional potential through shotgun metagenomics analysis [252]. Thereby, astronauts will be able in a near future to detect unwanted species in spaceflight habitats in real-time, but also catalogues of genes associated with unwanted microbial functions independently of the hosting species (genes involved in material degradation, biofilm persistence, virulence…). These metagenomic approaches have been developed with success in other biotopes such as the human gastrointestinal tract allowing for stratification of the population (e.g. enterotypes) in different responding groups and identifying biomarkers associated with specific functions [253] [254].

Microbial biofilms can also be used for some of their positive effects on crew health [255]. They are envisioned as a source of safe, fresh and valuable food to improve astronauts' health for long-term spatial missions. Several solid fermentation processes involving biofilms are/could be explored in this context e.g. miso and natto resulting from the biofilm formation of *Aspergillus oryzae* or *Bacillus subtilis* on cooked beans [256]. Kefir granules and relatives products are centimetre natural symbiotic communities composed of lactic acid bacteria and yeasts embedded in a dense and complex extracellular matrix [257]. Described as functional “super-organisms”, these spatially organised consortia are highly tolerant to environmental stress and could be multiplied for years during long-term missions with low resource requirements. Astronauts do not receive the same replenishment of microbes on a space flight as they do on earth [34]; fermented food along with probiotics can be used to prevent or restore gut microbiota dysbiosis. Another possible source of fresh food is the cultivation

888 of microalgae with nutritive interest (lipids, vitamines…). In order to limit the use of water and
889 energy, biofilm-based microalgae cultivation systems on surfaces are developed [158]. Alternatively,
890 bioprinting of microalgae cells with controlled patterns in hydrogels could allow the formation of a
891 synthetic biofilm with optimum exposure to light and nutrients [258] [259]. An important advantage
892 of these microorganism based-processes involving biofilms is that they could be adapted to recycle
893 organic matter from waste. More generally, biofilms are envisioned in various *in-situ* resource
894 utilization (ISRU) procedures in space travel such as biomining or bioregenerative life-support
895 systems [260].

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904 7. References

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Legends to Figures

Figure 1. Biofilms in spaceflight habitats. A) Mold-stained panels in the ISS hygiene area resulting from mould growth following contact with wet towels. Image: Mold species in dust from the International Space Station identified and quantified by mould-specific quantitative PCR [24]; B) Biofilm formation inside the condensate plumbing at the inlet to the Russian SRV-K condensate processor [25].

Figure 2. Different 3D structures obtained in terrestrial or microgravity induce different molecular diffusion. To illustrate this difference, we computed a reaction-diffusion process based on confocal microscopy images of Pseudomonas aeruginosa grown under 1 g or microgravity taken in [30]. A chemical component diffuses from a bulk source located in the upper boundary of the image with a heterogeneous diffusion coefficient that depends on the biofilm density: the higher the local bacterial density, the lower the local diffusion coefficient. The reactive process corresponds to the chemical consumption by the biofilm bacteria, the rate of which also varies according to the local bacterial density with Monod dependency. The steady state of the chemical density map is displayed, with isolines every 0.05 to better represent the distribution gradients. These different concentration maps induce different nutrient availability which in turn may impact the 3D biofilm structure and physiology.

Figure 3: Schematic view of surface colonisation by microorganisms. Microorganisms from the bulk are transported toward a surface through passive and active processes. After contact with the surface, cells can migrate individually or collectively and initiate the formation of a 3D structure called biofilm associated with emergent properties such as

extreme environmental stress tolerance. Subpopulations of cells can disseminate from this primary structure to initiate secondary biofilms with potential phenotypic and genetic evolution. Those microbial processes were formally described on earth and there is rising scientific evidence that many of them could be strongly affected under hyper- or micro-gravity conditions encountered in spaceflight missions.

Figure 4. We simulated the growth of a micro-alga biofilm subject to terrestrial or micro-gravity. The model is a mixture model [158] coupling a fluid dynamics model to a reaction-diffusion-convection model of biofilm dynamics including biomass growth and consumption of diffusive nutrients and CO2. Compared to [158], an additional force is added to the movement conservation equation modelling the gravity-dependent sedimentation as a net force between a gravitational force and a buoyant force [154]. We can observe that micro-gravity impacts the spatial structure of the biofilm and therefore the resulting substrate consumption and the overall biofilm growth.

Figure 5: (A) Cluego representation of common biological process grouping genes involved in similar pathways from BRIC-21 and BRIC-23 datasets. Each node corresponds to a GO (Gene ontology) term. Blue and red colours illustrated the contribution of genes upregulated in BRIC21 and BRIC23, respectively. The size of the node represents the term enrichment significance. The visualization was obtained with the Cluego Plugin for Cytoscape 3.9.1. (10.1093/bioinformatics/btp101, 10.1101/gr.1239303). (B) Venn diagram showing genes of the ResD and Rex regulons significantly downregulated in the BRIC-21 and BRIC-23 missions (data from [187]).

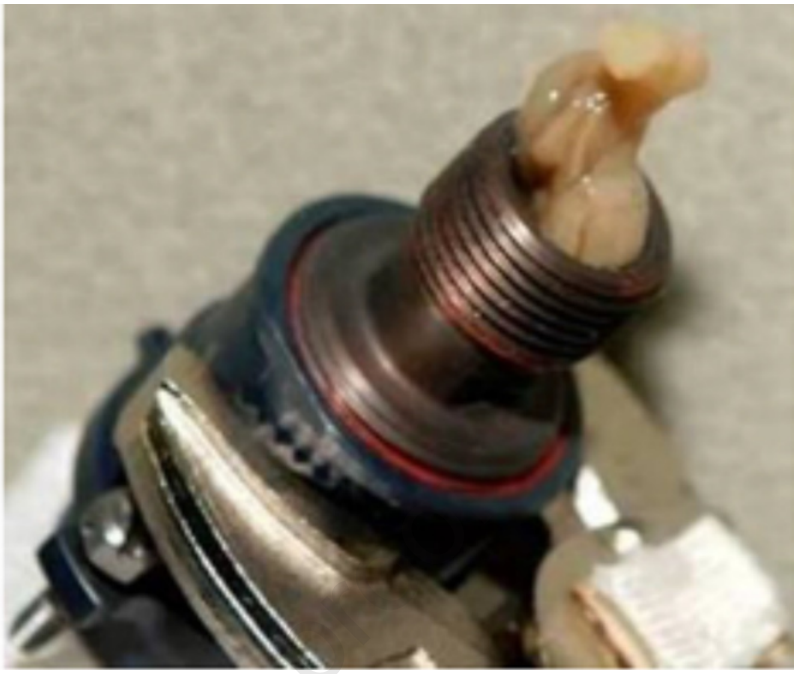
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Table 1: *Surface modifications to prevent biofilm formation and their mode of action.*

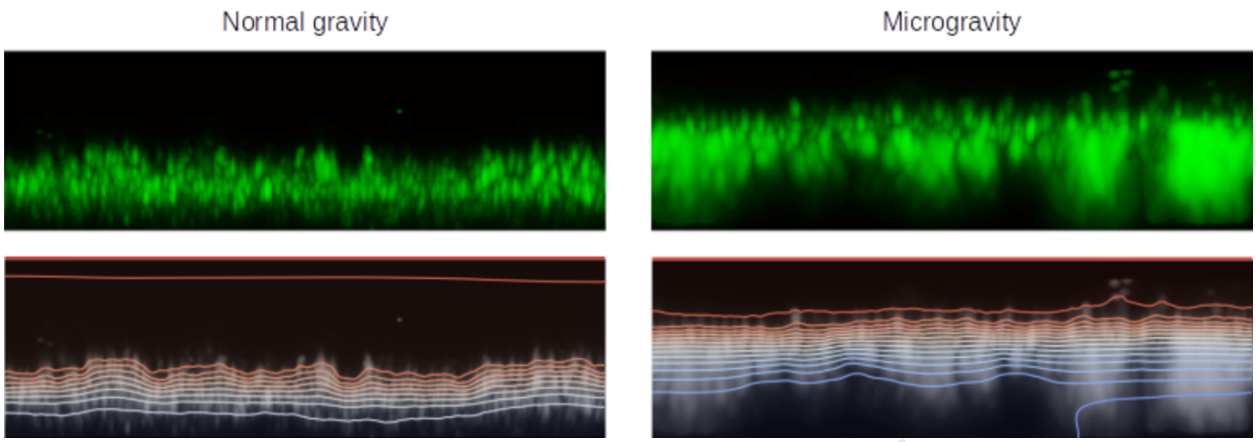
Coating type	Coating agent	Description	Action mechanism	References
Polymeric films (organic, synthetic, mixed)	Poly(2-alkylacrylic acids), layer by layer (LdL)	Agents released in response to environmental acidification due to bacterial metabolism	Release-killing	[217]
Metal ions (copper, argent, titanium)	Octadecylamine capped Cu/reduced graphene oxide	Enhancing surface hydrophobicity	Anti-adhesion	[218]
Metal ions (copper, argent, titanium)	Silver oxide film	Photocatalytic antimicrobial surfaces	Contact-killing	[219]
Liquid films	Liquid-infused structured surfaces	Imbibition of porous surfaces with surfactants	Anti-adhesion	[220]
Antimicrobial peptides (AMPs) grafting	Polydopamine peptide coating	Polydopamine coating to immobilize AMPs on surfaces	Contact-killing	[221]
Quorum Quenching enzymes grafting	Acylase and α-amylase coating	Degradation of Quorum Sensing signals	Anti-adhesion	[222]
Nanoparticles grafting	Zinc oxide nanoparticle	Immobilization of antibacterial nanoparticles on surfaces	Contact-killing	[223]

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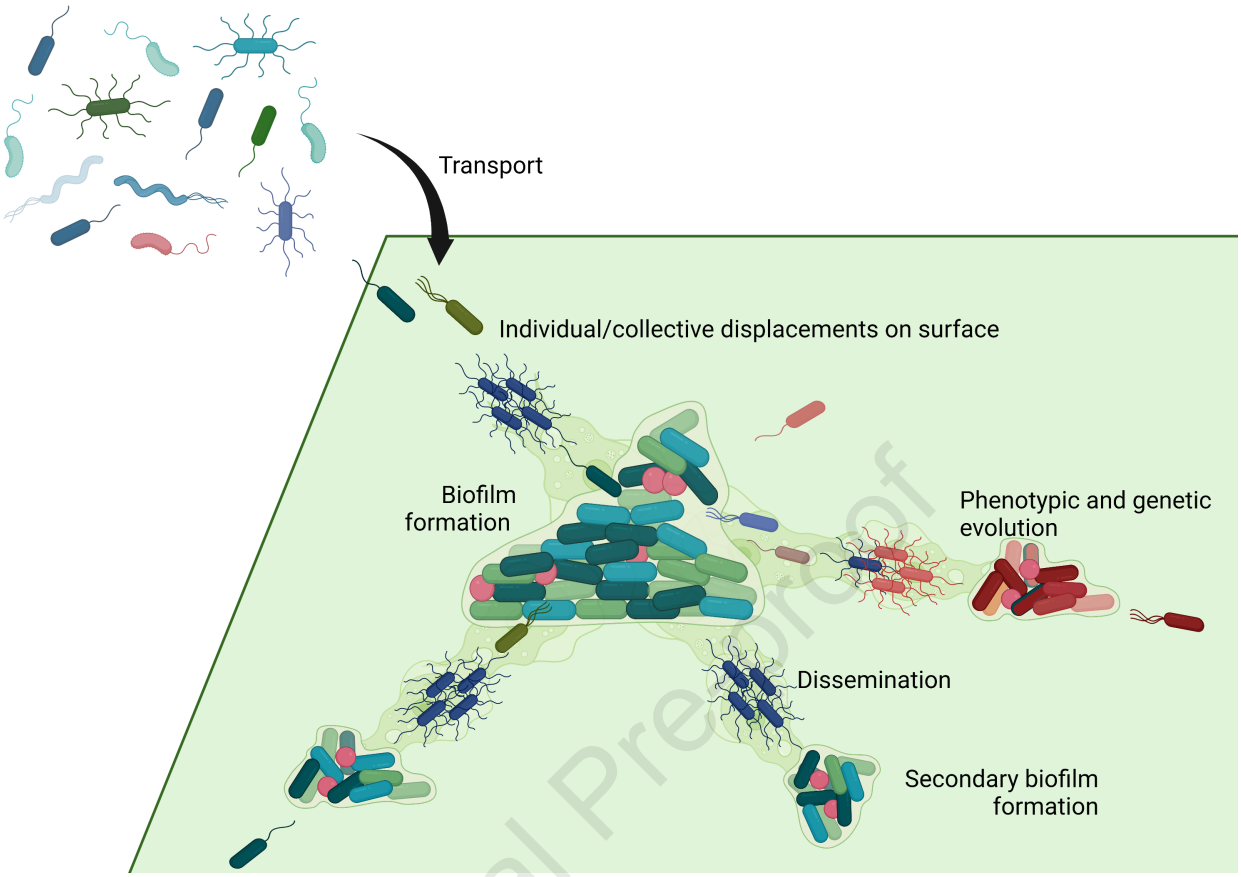




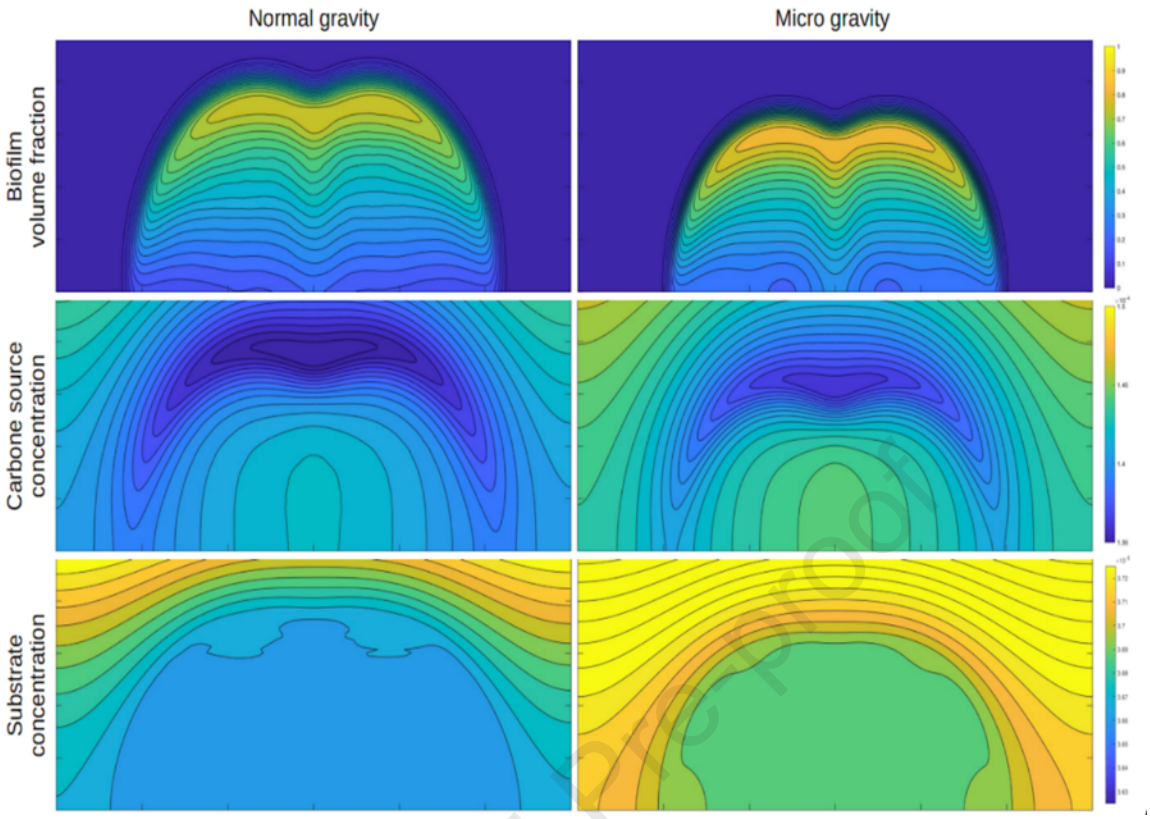
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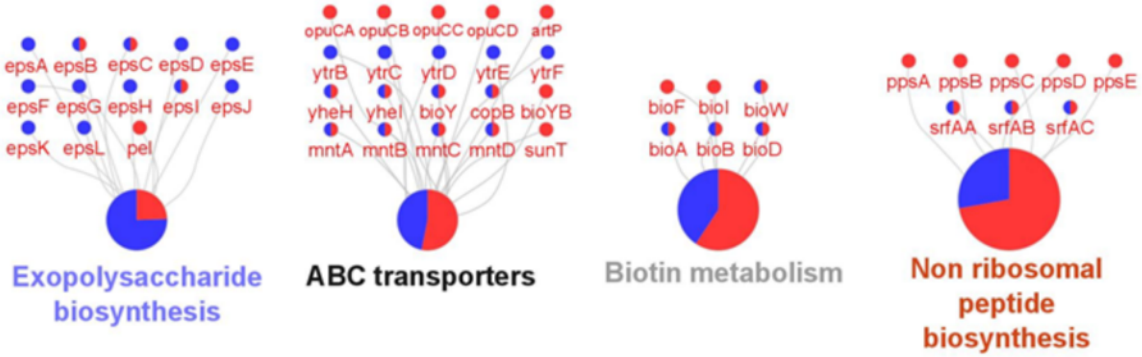
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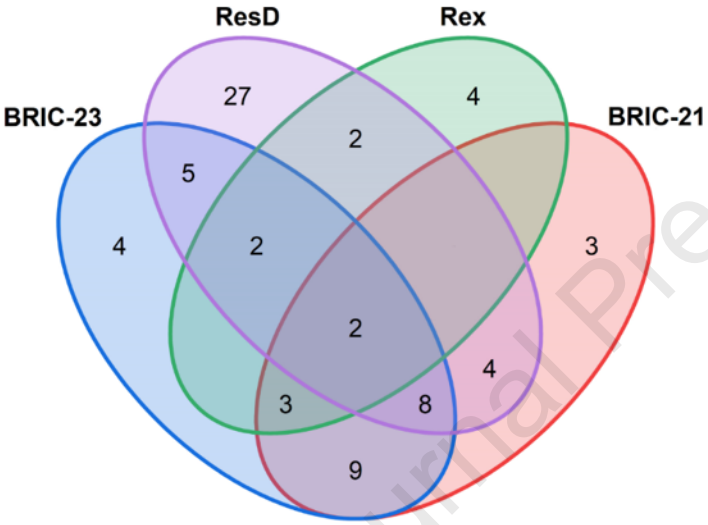


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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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