Effect of arsenite on swimming motility delays surface colonization in \textit{Herminiimonas arsenicoxydans}

M. Marchal,\textsuperscript{1} R. Briandet,\textsuperscript{2} S. Koechler,\textsuperscript{1} B. Kammerer\textsuperscript{1} and P. N. Bertin\textsuperscript{1}

\textsuperscript{1}Génétique Moléculaire, Génomique et Microbiologie, UMR7156 CNRS & Université de Strasbourg, Strasbourg, France
\textsuperscript{2}INRA, UMR1319 MICALIS, Massy, France

\textit{Herminiimonas arsenicoxydans} is a Gram-negative bacterium able to detoxify arsenic-contaminated environments by oxidizing arsenite \([\text{As}(\text{III})]\) to arsenate \([\text{As}(\text{V})]\) and by scavenging arsenic ions in an extracellular matrix. Its motility and colonization behaviour have been previously suggested to be influenced by arsenite. Using time-course confocal laser scanning microscopy, we investigated its biofilm development in the absence and presence of arsenite. Arsenite was shown to delay biofilm initiation in the wild-type strain; this was partly explained by its toxicity, which caused an increased growth lag time. However, this delayed adhesion step in the presence of arsenite was not observed in either a swimming motility defective \(fliL\) mutant or an arsenite oxidase defective \(aoxB\) mutant; both strains displayed the wild-type surface properties and growth capacities. We propose that during the biofilm formation process arsenite acts on swimming motility as a result of the arsenite oxidase activity, preventing the switch between planktonic and sessile lifestyles. Our study therefore highlights the existence, under arsenite exposure, of a competition between swimming motility, resulting from arsenite oxidation, and biofilm initiation.

\section*{INTRODUCTION}

For many years human activities have resulted in a huge accumulation of toxic elements in the biosphere, including heavy metals and metalloids. These elements, unlike most organic contaminants, are not degradable, leading to their persistence in the environment. Metallic pollution of waters has also gradually become a major concern worldwide, and particular attention has been given to arsenic, one of the most toxic elements among the metalloids and metallic ions currently found in the environment. This metalloid can have multiple health effects, including diabetes, hypertension and skin lesions. Furthermore, long-term exposure has been associated with the development of skin and internal cancers (Abernathy \textit{et al.}, 1999).

Inorganic forms of arsenic are the dominant species in waters, arsenite \([\text{As}(\text{III})]\) being considered as more toxic than arsenate \([\text{As}(\text{V})]\) (Oremland & Stolz, 2003). The financial and ecological costs of the chemical techniques currently used to remove arsenic from waters prompted the development of biological processes (Lièvremont \textit{et al.}, 2009). In this respect, bacteria possessing the arsenite oxidase enzyme, which oxidizes As(III) to the less toxic and also less soluble form As(V), are of particular interest. \textit{Herminiimonas arsenicoxydans} is an arsenite-oxidizing bacterium originally isolated from the activated sludge of an industrial water treatment plant contaminated with heavy metals and arsenic (Muller \textit{et al.}, 2006). This Gram-negative bacterium is also able to scavenge arsenic in an exopolysaccharide matrix (Muller \textit{et al.}, 2007). Both properties make \textit{H. arsenicoxydans} of particular interest for use in bioremediation.

Physiological tests and proteomic analyses have revealed that the colonization abilities of \textit{H. arsenicoxydans} are specifically affected by As(III) under planktonic growth conditions. As(III) was shown to induce both motility and the synthesis of extracellular polysaccharides (Muller \textit{et al.}, 2007; Weiss \textit{et al.}, 2009), known to play a role in biofilm formation and development. Extracellular polymeric substances (EPS) can mediate adhesion to surfaces and cohesion of biofilm structures, representing the matrix in which bacteria are embedded (Flemming & Wingender, 2001). Moreover, EPS are known for their protective role against various bactericidal compounds, including metallic ions (Harrison \textit{et al.}, 2007). In particular, they have been shown to trap arsenic ions in \textit{H. arsenicoxydans} (Muller \textit{et al.}, 2007).
The relationship between biofilm and flagellar motility is diverse among bacterial species, and motility can be involved at different levels (Verstraeten et al., 2008). Firstly, motility can play an essential role in biofilm formation, promoting adhesion to the surface (Pratt & Kolter, 1998), biofilm maturation (Klausen et al., 2003a, b; Todhanakasem & Young, 2008) and/or dispersal processes (Sauer et al., 2002; Tolkner-Nielsen et al., 2000). Flagellar motility can also compete with biofilm formation when bacteria have to select between sessile and planktonic lifestyles, both processes involving similar pathways and being regulated by environmental factors (Verstraeten et al., 2008).

Even though the role of motility in the early stages of biofilm formation is difficult to predict, a good knowledge of the colonization behaviour of a strain is essential to develop a bioremediation strategy. The aim of the present study was to explore both biofilm initiation and development in H. arsenicoxydans and to elucidate the role of motility and the effects of arsenite in these steps. For this purpose, biofilm formation of the wild-type strain, a Δflil mutant affected in its swimming properties (Muller et al., 2007) and an ΔaooX mutant defective in its arsenite oxidase activity (Muller et al., 2003) was compared using confocal and epifluorescence microscopy in the absence and in the presence of subinhibitory As(III) concentrations. We observed delayed biofilm formation in the presence of arsenite in the wild-type strain, but not in either the motility or the arsenite oxidase defective mutant.

**METHODS**

**Bacterial strains and culture conditions.** *Herminiimonas arsenicoxydans*, a β-proteobacterium, was isolated from industrial activated sludge contaminated with heavy metals and arsenic, and shown to be resistant to up to 5 mM As(III) (Weeger et al., 1999). The Δflil mutant, shown to be defective in swimming motility (Muller et al., 2007), was obtained by in-frame insertion of a mini-Tn5::lacZ2 in the 88th codon of the flil gene (Carapito et al., 2006). The ΔaooX mutant, defective in arsenite oxidase activity, was obtained by insertion of a mini-Tn5::lacZ2 in the 335th codon of the aooX gene (Muller et al., 2003). The mini-Tn5::lacZ2 does not carry transcriptional terminators (de Lorenzo et al., 1990). Strains were cultivated at 25 °C in a chemically defined medium (CDM; Muller et al., 2006). Arsenic was added to the medium to the desired concentration from sterile stocks of 666.7 mM (50 000 p.p.m.) of the metalloid in deionized water, from either NaAsO2 concentration from sterile stocks of 666.7 mM (50 000 p.p.m.) of the resistant to up to 5 mM As(III) (Weeger et al., 1999). The Δflil mutant was checked by RT-PCR in order to confirm that only the flil gene was inactivated by the mini-Tn5 and not the full flilMNPOR operon. RT-PCRs were performed on 200 ng of total RNA for a total reaction volume of 25 μl using SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen), according to the manufacturer’s recommendations. Primers were used as follows: 5′-ACAGGAAGATTGTGATGCTTTTAA-3′ and 5′-ATGTACTCGAATTCAACCGGATAG-3′ (forward and reverse primers, respectively, for the flilM gene).

**Assessment of cell wall properties.** The surface properties of the strains were investigated using the MATS (microbial adhesion to solvents) method (Bellon-Fontaine et al., 1996) on 1 ml of an exponential culture (OD600 0.15 on a Spectronic 20 Genesis spectrophotometer) harvested and resuspended in 1 ml 150 mM NaCl Affinity tests were performed in chloroform and hexadecane solvents (Sigma).

**Transmission electron microscopy.** To assess the presence of a flagellum on the bacterial cells, exponential-phase cultures were carefully deposited on Formvar-coated nickel grids, without any staining. Grids were examined using a Hitachi H600 transmission electron microscope at 75 kV and photographed using a Hamamatsu ORCA-HR camera with the AMT software (Advanced Microscopy Techniques).

**Confocal laser scanning microscopy (CLSM).** Cultures were performed in microscopy-quality 35 mm polystyrene dishes (ibiTreat low 35 mm μ-Dish, Ibidi, Integrated BioDiagnostics) as follows. Samples (1.5 ml) of growth medium supplemented or not with 0.67 mM As(III) were inoculated with 15 ml of an exponential-phase culture grown to an OD600 of 0.15 on a Spectronic 20 Genesis spectrophotometer. Incubation was performed at 25 °C under stagnant culture conditions. The medium was renewed every day to avoid growth limitation due to nutrient depletion. Before staining, samples were gently rinsed with 1 ml 150 mM NaCl to remove loosely adherent planktonic cells. Cells were labelled with the cell-permeable dye SYTO9 (Molecular Probes, Invitrogen), a fluorescently labelled lectin, Alexa Fluor 633 conjugated concanavalin A (Molecular Probes, Invitrogen), which selectively binds α-mannopyranosyl and α-glucopyranosyl residues, was used to visualize biofilm exopolysaccharides. Fifty microlitres of stock solution of each dye (6 μM SYTO9 and 1 mg Alexa Fluor 633 conjugated concanavalin A ml−1) were deposited on the cells for 15 min. Z-stacks of horizontal-plane images of biofilms were acquired with a step of 1 μm using multimodal CLSM (Leica TCS SP2 AOBS, Leica Microsystems) with a ×63 (1.4 NA) oil-immersion lens. Images were recorded at an excitation wavelength of 488 nm and 633 nm and emission wavelengths from 500 to 530 nm and from 657 to 757 nm for SYTO9 and Alexa Fluor 633 conjugated concanavalin A, respectively. Simulated 3D fluorescence projections were generated using IMARIS 7.0.0 software (Bitplane). The surface coverage was quantified using NIH ImageJ analysis software (http://rsbweb.nih.gov/ij/). A manual threshold was applied to binarize images. For each experiment, six random microscope fields from two replicates were analysed.

**Epifluorescence microscopy.** Cultures for adhesion assays were performed in microscopy-quality 35 mm polystyrene dishes as described above. Attached cells were stained with SYTO9 as described above. Digital images of the attached bacteria were captured at a magnification of ×1000 under oil immersion using a Leica DM 4000 B epifluorescence microscope equipped with a Leica DFC300 FX digital camera (Leica Microsystems). Images were recorded at an excitation...
wavelength of 488 nm and emission wavelengths from 500 to 550 nm. The surface coverage was quantified as described above for CLSM.

**Statistical analysis.** The ANOVA test was used for comparing strain growth parameters, adhesion to solvents and evaluating the strain surface coverage. Significance was set at $P<0.05$.

## RESULTS

### Three-step biofilm development in *H. arsenicoxydans*

To investigate biofilm formation in *H. arsenicoxydans*, the strain was cultivated in microscope-quality 35 mm polystyrene dishes, in static conditions. Biofilm development was followed using multimodal CLSM. The cells were stained with SYTO9 and the polysaccharides surrounding the cells were marked with Alexa Fluor 633 conjugated concanavalin A (ConA). This double staining highlighted, after 24 h incubation, small microcolonies (7.2 ± 1.2 µm height) formed by globular structures of a saccharidic nature, i.e. marked by the ConA, around which bacterial cells aggregated (Fig. 1). In parallel, the biofilm surface coverage was measured as a function of time (Figs 2 and 3; see also Supplementary Table S1, available with the online version of this paper). The same surface coverage results were obtained using epifluorescence microscopy (data not shown). This approach highlighted a three-step

![Fig. 1. Four-day-old biofilm of *H. arsenicoxydans*. (a) Single section of the biofilm surface coverage. Examples of microcolonies, small bacterial aggregates, are circled. (b) Single section of a microcolony. (c) Isosurface view of the microcolony depicted in (b) by IMARIS 7.0.0 software. CLSM images were acquired with optical section separation (z-interval) of 1 µm. The IMARIS reconstruction was obtained from a 15-section stack. Cells were stained with SYTO9 (green) and the extracellular matrix with Alexa Fluor 633 conjugated concanavalin A (red).](image-url)
biofilm development, i.e. the attachment of the cells to the surface, the formation of small globular structures and the biofilm detachment 48 h later. Some microcolonies remained on the surface after 72 h.

Involvement of motility in the surface colonization process

The biofilm formation of a ΔfliL mutant, defective in swimming motility, was investigated in order to assess the role of flagellar motility in *H. arsenicoxydans* biofilm development. This mutant was obtained by in-frame insertion of a mini-Tn5::lacZ2 in the 88th codon of the fliL gene, and fliL transcription has been previously shown to be induced under arsenite stress. This gene being the first gene of the fliLMNOPQR operon, the presence of fliM transcripts in the *H. arsenicoxydans* ΔfliL mutant was verified by RT-PCR (data not shown), ensuring that this insertion did not affect the transcription of other operon genes, consistent with the fact that the mini-Tn5::lacZ2 has no transcriptional terminators. The presence of a flagellum, which influences the membrane properties of the cell, was confirmed by transmission electron microscopy (Supplementary Fig. S1). The surface properties of the ΔfliL mutant were also unaffected by the mutation (Supplementary Table S2). Furthermore, the mutation did not affect the planktonic growth of the strain (Table 1).

The biofilm development of the ΔfliL mutant was followed by CLSM. Globular microcolonies, similar to those observed in the wild-type strain biofilm, were present and the surface colonization kinetics appeared to be similar to that of the wild-type strain (Fig. 3, Supplementary Table S1 and data not shown). The same surface adhesion results were obtained using epifluorescence microscopy (data not shown). Based on these observations, we concluded that, in the absence of arsenite, swimming motility does not act, positively or negatively, in *H. arsenicoxydans* biofilm development.

**Effect of arsenite on biofilm development**

We then investigated *H. arsenicoxydans* biofilm formation under arsenite stress. The biofilm structure, i.e. the globular microcolonies, of the wild-type strain was not affected by As(III) (data not shown). However the three-step biofilm development was delayed by 24 h (Fig. 2, Fig. 3 and Supplementary Table S1), even when the strain was pre-cultivated with As(III) (data not shown). The same surface coverage results were obtained using epifluorescence microscopy (data not shown). In order to explain this observation, the planktonic growth of the strain was

![Fig. 2. *H. arsenicoxydans* biofilm surface coverage as a function of time. Cultures were performed in the absence of arsenic (○) and in the presence of 0.67 mM As(III) (●). The values are means ± 1 SD for data from six images (three images from two replicate biofilms). NIH ImageJ analysis software (http://rsbweb.nih.gov/ij/) was used to assess surface coverage by the cells stained with SYTO9.](http://mic.sgmjournals.org)

![Fig. 3. Biofilm surface coverage of the wild-type strain (WT) and the ΔfliL and ΔaoxB mutants in the absence or presence of 0.67 mM As(III). The data are depicted as box plot diagrams. Box plots portray median, quartiles, adjacent values and outlying values for data from six images (three images from two replicate biofilms).](http://mic.sgmjournals.org)
followed in the absence and in the presence of 0.67 mM As(III) (Table 1). The results showed a marked increase in lag time in the presence of arsenite, from 1.5 h to about 13 h; this was seen even when the strain was pre-cultivated with 0.67 mM As(III) (data not shown). This observation could partly explain the delayed surface colonization in the presence of arsenite.

Role of motility in biofilm initiation in the presence of arsenite

Since As(III) is known to induce swimming motility in *H. arsenicoxydans*, we hypothesized that it could favour swimming instead of surface colonization. To address this hypothesis, we investigated the early stages of biofilm formation of the *ΔfliL* mutant in the presence of arsenite. Indeed, the mutant displayed an adhesion rate at 24 h significantly higher than that of the wild-type strain (*P*<0.05) (Fig. 3, Supplementary Table S1 and Supplementary Fig. S2). The same surface coverage results were obtained using epifluorescence microscopy (data not shown). This observation could partly explain the delayed surface colonization in the presence of arsenite.

### Table 1. Growth parameters of the wild-type strain (WT) and *ΔfliL* and *ΔaoxB* mutants as a function of arsenite concentration

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth parameter*</th>
<th>Arsenite concentration (mM):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>WT</td>
<td>g (h)</td>
<td>3.2±0.1</td>
</tr>
<tr>
<td></td>
<td>L (h)</td>
<td>1.5±0.9</td>
</tr>
<tr>
<td><em>ΔfliL</em></td>
<td>g (h)</td>
<td>3.2±0.1</td>
</tr>
<tr>
<td></td>
<td>L (h)</td>
<td>2.7±0.1</td>
</tr>
<tr>
<td><em>ΔaoxB</em></td>
<td>g (h)</td>
<td>3.3±0.1</td>
</tr>
<tr>
<td></td>
<td>L (h)</td>
<td>2.5±0.4</td>
</tr>
</tbody>
</table>

*g*. generation time; L, lag time.

Induction of swimming motility through arsenite oxidase activity

To test if this swimming motility induction was dependent on arsenite oxidase activity, we investigated the surface colonization behaviour of a *ΔaoxB* mutant. This strain carries a Tn5 transposon insertion in the gene coding for the large subunit of the arsenite oxidase and displays the wild-type cell surface properties (*P*>0.05) (Supplementary Table S2). Using epifluorescence microscopy, we showed that, in the absence of arsenite, the mutant exhibited the wild-type surface colonization behaviour. Interestingly, under arsenite exposure, the surface colonization of this mutant was not delayed by 24 h (Fig. 3, Supplementary Table S1 and Supplementary Fig. S2). Under arsenite stress, this *ΔaoxB* mutant exhibited a planktonic generation time similar to and a lag time of 2.5 h shorter than those of the wild-type strain (Table 1). However, this lag time difference was insufficient to explain the 24 h difference in the surface colonization behaviour between the wild-type strain and the *ΔaoxB* mutant under arsenite exposure. According to these data, we concluded that this colonization difference was correlated with arsenite oxidase activity.

Effects of As(V) on biofilm initiation

To confirm that the delayed surface adhesion we observed in the presence of As(III) in the wild-type strain was specific to the arsenite oxidase activity and not to the presence of As(V), the final product of the enzymic activity, we investigated *H. arsenicoxydans* biofilm initiation in the presence of 0.67 mM As(V) using epifluorescence microscopy. Even though this As(V) concentration induced a planktonic lag time of 13±0.4 h similar to that induced by 0.67 mM As(III), its presence did not delay biofilm initiation (Supplementary Table S1). This result supports our conclusion that arsenite oxidase activity, and not As(V), was responsible for the delayed adhesion observed in the presence of As(III).

**DISCUSSION**

In the natural environment, most bacteria live in organized surface communities called biofilms. These aggregated cells are embedded in an exopolymeric matrix which protects them against various environmental stresses, e.g. desiccation, oxygen and antibacterial compounds (Costerton et al., 1995; O’Toole et al., 2000). Biofilm formation also represents an important survival mechanism; biofilm-forming strains show increased resistance to antibacterial compounds such as antibiotics and metallic ions. This increased resistance depends on multiple factors, including the physical barrier formed by the exopolymeric matrix (Harrison et al., 2007). The biofilm lifestyle is also advantageous in stressful conditions as compared to the planktonic one. The arsenic-resistant bacterium *H. arsenicoxydans* has been shown to synthesize a thicker exopolysaccharide matrix in the presence of subinhibitory As(III) concentrations (Muller et al., 2007). These transmission electron microscopy observations have been further supported by proteomic and quantitative mRNA analysis, indicating the accumulation of two proteins potentially involved in exopolysaccharide synthesis – a polysaccharide biosynthesis protein CapE (HEAR1147) and a phosphomannomutase producing a precursor for alginate polymerization (HEAR2721) – and a twofold induction of a
gene coding an EpsF-like protein (HEAR0715) in the presence of As(III) (Muller et al., 2007; Weiss et al., 2009). As an exopolysaccharidic matrix is often linked to biofilm structures (Flemming & Wingender, 2001), we hypothesized that subinhibitory As(III) concentrations induce biofilm formation in H. arsenicoxydans.

The staining used for biofilm exopolysaccharides in the present study did not indicate an increased amount of exopolysaccharide in the presence of As(III), contrary to what has been previously described under planktonic growth conditions (Muller et al., 2007). This result may indicate a difference in regulation of exopolysaccharide synthesis between the two lifestyles. However, in the stagnant culture conditions tested in the present study, even though arsenite did not affect the biofilm structure, we showed that its presence delayed biofilm initiation. The toxic effect of arsenite on growth could partly explain this late adhesion. However, the surface colonization kinetics may also be regulated by other factors, including motility. H. arsenicoxydans possesses a polar flagellum and is also capable of swimming motility (Muller et al., 2007). The role of swimming motility in biofilm initiation and development was investigated using a non-motile ΔfilL mutant previously obtained by insertion of a mini-Tn5 (Carapito et al., 2006; Muller et al., 2007). The filL gene is the first gene of the filLMNOPQR operon, belonging to the class 2 genes, and encoding flagellar structural proteins implicated in the specification of the basal body and hook structure (Macnab, 2004). We showed that the mutant displays wild-type surface properties, including the presence of a flagellum, which is known to influence the surface properties of the cell and can act as an adhesive appendage (Kirov et al., 2004; Nejidat et al., 2008). In the absence of arsenite, the mutant also exhibited wild-type biofilm formation kinetics. Based on these observations and according to the mutant phenotype, we conclude that in the absence of arsenite, swimming motility has neither positive nor negative effects on H. arsenicoxydans biofilm development, a process that may require other motility forms, like twitching (Klausen et al., 2003b). However, our results do not exclude a role of motility in the initiation of this process, i.e. in the switch between planktonic and sessile lifestyle. As H. arsenicoxydans swimming motility has previously been shown to be specifically induced by arsenite stress (Muller et al., 2007), we hypothesized that As(III) could favour swimming instead of surface colonization, which would explain the 24 h delay in surface colonization observed in the presence of arsenite. This hypothesis is supported by the fact that, in the presence of As(III), the ΔfilL mutant exhibited a prompter initial attachment than the wild-type strain. As the filL gene has been previously shown by β-galactosidase assays and proteomic analysis to be induced in the presence of arsenite, like the swimming motility (Carapito et al., 2006; Weiss et al., 2009), we propose that the presence of As(III) favours swimming motility through the filL gene, resulting in a delayed switch between motile and sessile lifestyles, a transition that is essential in biofilm initiation.

In numerous bacteria, such as Pseudomonas aeruginosa, Salmonella Typhimurium and Vibrio cholerae, the switch between motile and sessile lifestyles has been shown to be regulated by a global second messenger, bis-(3',5')-cyclic dimeric guanosine monophosphate (c-di-GMP) (Barraud et al., 2009; Jonas et al., 2010; Kolter & Greenberg, 2006; Pratt et al., 2007). Its intracellular concentration depends on diguanylate cyclase activities, associated with proteins possessing GGDEF domains, and the c-di-GMP-specific phosphodiesterase activities, linked to proteins presenting EAL domains. High c-di-GMP cellular levels induce biofilm formation while low levels promote cell motility (Hengge, 2009). Transcriptional profiling of H. arsenicoxydans has been recently performed under arsenite exposure (Koechler et al., 2010). The transcription of a gene coding for a hypothetical diguanylate cyclase carrying a GGDEF domain protein was found to be significantly inhibited (2.5-fold) in the presence of arsenite (J. Cleiss-Arnold, personal communication). This gene (HEAR1864) is located directly upstream of the cluster II flagellar genes (HEAR1866 to HEAR1904), whose transcription has been shown to be induced by arsenite (Koechler et al., 2010). These observations support our hypothesis, i.e. an induction of swimming motility at the expense of biofilm formation in the presence of arsenite.

Flagellar motility is a process that is also considered as a defence mechanism in hostile environments. It allows bacteria to reach more favourable niches, giving them an ecological advantage (Eberl et al., 1999; Harshey, 2003). However, since As(III) has been previously shown to induce positive chemotaxis in H. arsenicoxydans, the fact that motility is induced in this bacterium in the presence of As(III) could not be explained by this hypothesis. Alternatively the arsenite-dependent motility may be the result of arsenite oxidase activity: As(III) has been shown to promote increased motility in H. arsenicoxydans wild-type but not in arsenite oxidase defective strains (Koechler et al., 2010; Muller et al., 2007). Indeed, the arsenite oxidase defective mutant tested in the present study did not exhibit delayed adhesion under As(III) exposure. Because in the presence of As(V), the final product of the arsenite oxidase activity, the wild-type strain did not exhibit delayed surface adhesion, we conclude that the delayed biofilm initiation we observed under As(III) exposure was specifically dependent on the arsenite oxidase activity.

In conclusion, our observations show that As(III) has a more complex effect on surface colonization than previously thought. In H. arsenicoxydans, flagellar motility plays a key role in the switch between planktonic and sessile lifestyles, inhibiting the initiation of biofilm formation. Surprisingly, under arsenite stress, H. arsenicoxydans favours swimming motility, which results from the arsenite oxidase detoxification activity. The swimming motility resulting from this activity may be indicative of an attempt by the cells to transform their environment before they initiate a biofilm.
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