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Attachment to Stainless Steel by Mir Space Station Bacteria Growing Under Modeled Reduced Gravity at Varying Nutrient Concentrations

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Attachment to stainless steel by Mir Space Station bacteria growing under modeled reduced gravity at varying nutrient concentrations

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ABSTRACT

Four bacterial isolates (*Chryseobacterium* sp., *Pseudomonas fluorescens* and two *Stenotrophomonas maltophilia* isolates) originally isolated from the water system aboard the Mir Space Station were grown in two concentrations of nutrient broth under modeled reduced gravity using clinorotation. Sampling was performed over a 7 day period and planktonic cells were enumerated using 4′,6-diamidino-2-phenylindole (DAPI), while those attached to stainless steel were enumerated using the LIVE/DEAD® *Bac*Light™ kit and DAPI. On some of the sampling days for all the isolates, planktonic cell counts were higher under modeled reduced gravity as compared with the normal gravity controls. In contrast, the number of cells of *P. fluorescens* and one *S. maltophilia* isolate attached to the stainless steel disks was higher under modeled reduced gravity as compared with normal gravity, whereas no such differences were observed for *Chryseobacterium* sp. and the other *S. maltophilia* isolate. Differences in motility among isolates appeared to influence the growth of planktonic cells under modeled reduced gravity but did not appear to be related to biofilm formation.

INTRODUCTION

Many studies focusing on responses of bacteria to reduced gravity have investigated the growth of planktonic bacterial cells under actual microgravity conditions (Gasset *et al.*, 1994; Kacena & Todd, 1997; Kacena *et al.*, 1997, 1999a,b,c; Klaus *et al.*, 1997; Brown *et al.*, 2002). Alternatively, reduced gravity conditions can be modeled using clinorotation, where a cell in a rotating vessel experiences conditions of randomized gravitational vectors; this approach provides a cost-effective option that allows researchers to conduct a larger number of experiments (Gao *et al.*, 1997; Hammond & Hammond, 2001; Klaus, 2001; Nickerson *et al.*, 2003). Basically with clinorotation, a slow-turning lateral vessel (STLV) or high-aspect rotating vessel revolves around its horizontal axis and the cells contained in the vessels are maintained in a perpetually freefall environment. A number of studies on the growth of bacteria have been performed using clinorotation (Huitema *et al.*, 2002; England *et al.*, 2003; Nickerson *et al.*, 2003; Baker & Leff, 2004) and similar results can be obtained using clinorotation compared with actual microgravity conditions (Kacena *et al.*, 1999a).

Although responses of planktonic cells suspended in liquid medium have been examined in several studies (e.g. England *et al.*, 2003; Baker & Leff, 2004), few studies have examined biofilm formation by bacteria under microgravity conditions (McLean *et al.*, 2001). The purpose of our study was to examine the attachment of bacterial cells, from a modeled reduced-gravity environment, to stainless steel disks. These studies were performed on bacteria that had previously been exposed to a microgravity environment, rather than standard "laboratory" strains that have been used in most other studies (Gasset *et al.*, 1994; Kacena & Todd, 1997; Klaus *et al.*, 1997; Kacena *et al.*, 1999a,b,c; Brown *et al.*, 2002). Specifically, four isolates from water systems of the Mir Space Station were examined because the conditions in this environment may have selected for strains or species with particular responses to reduced gravity.

MATERIALS AND METHODS

Many bacterial isolates were recovered from the Mir Space Station water in the reservoir tank or recycled water (Pierson, 2001). For this study, four isolates from the Mir water system were provided by the U.S. National Aeronautics and Space Administration (NASA). They were previously identified using 16 S rRNA gene sequencing and biochemical tests as *Chryseobacterium*

sp. (Genbank accession number AY756725), *Pseudomonas fluorescens* (Genbank accession number AY756717) and two different isolates of *Stenotrophomonas maltophilia* (Genbank accession numbers AY756720 and AY756724; Song & Leff, 2005).

The STLVs (Synthecon, Houston, TX) used in this study were modified with four plug inserts in the lid of each vessel. Each plug had three steel wire prongs to which stainless disks can be attached and removed. Planktonic cells in the vessels were exposed to modeled reduced gravity, while the faces of the disks rotated with the STLV and thus were subject to normal gravity effects. The steel disks were centrally positioned between the bottom and top faces of the STLV; plugs to which the disks were attached were on top of the control vessels (which were horizontally rotated) and to the side on those rotated around the vertical axis (to model reduced gravity conditions). Prior to use, the steel disks (diameter = 1 cm) were placed into acetone for 10 min, followed by washing in isopropanol for 5 min as described (Venkateswaran *et al.*, 2001). The STLVs, including the steel disks, were autoclaved at 110 °C for 30 min, as recommended by the manufacturer (Synthecon). Four STLVs were completely filled with 240 ml of 0.2% or 0.002% (w/v) nutrient broth (NB) and then inoculated with the target organism as described by Baker & Leff (2004). The STLVs were rotated around the horizontal axis on a rotary cell culture system (RCCS; Synthecon) at 40 r.p.m. (centrifugal force equivalent to 80.7 cm/s^2) to simulate a modeled reduced-gravity environment. Normal gravity controls were run simultaneously with four STLVs on the RCCS rotating around the vertical axis at 40 r.p.m.

Planktonic samples were collected from the STLVs after 0, 24, 48, 72 and 168 h, and preserved with 1 volume of sample : 3 volumes of preservative (1 : 1, 8% (w/v) paraformaldehyde : 1 × phosphate-buffered saline (PBS), pH 7.2) and stored at 4 °C. Bacteria were enumerated using 4′,6-diamidino-2-phenylindole (DAPI) as described (Porter & Feig, 1980).

Bacteria were recovered from the steel disks after 24, 48, 72 and 168 h. The steel disks were gently washed in filtered sterile distilled water and then placed into a stomacher bag containing 1.5 ml filtered sterile distilled water and 2.25 ml of 1 × PBS (pH 7.2). They were then placed into a stomacher 80 (Seward Ltd, London, UK) for 2 min at medium setting. Stomachers have previously been shown to be the most effective method for the removal of bacterial biofilms by comparison with other methods (Gagnon & Slawson, 1999). The advantages of using stomaching rather than sonication were that more bacteria were recovered and cells were not misshapen due to the disruptiveness of the technique (data not shown). Afterwards, 1 ml was removed and bacteria were enumerated using the LIVE/DEAD® *Bac*Light™ kit as described (Boulos *et al.*, 1999). To the remaining solution, 1.8 ml of 8% (w/v) paraformaldehyde was added and stored at 4 °C. These bacteria were enumerated using DAPI. The number of cells attaching per sqare centimeter of steel disk was determined. In addition, another calculation was performed to account for differences in planktonic cell abundance under modeled reduced gravity as compared with normal gravity. For this calculation, the total number of cells attaching to each steel disk was divided by the total number of planktonic cells in a given STLV. This allows us to account for differences in the numbers of attached cells between treatments that were caused by differences in the number of planktonic cells available to colonize the disks.

The data were analyzed by one-way analysis of variance (ANOVA) using an SPSS statistical package for Windows (SPPS Inc., Chicago, IL, USA). *Post hoc* tests were performed using Tukey's test. Significant differences were defined as cases where $P < 0.05$.

RESULTS

For each bacterial isolate grown in 0.2% NB, there were often significantly higher numbers of planktonic cells (based on DAPI staining) under modeled reduced gravity (MRG) as compared with normal gravity (Fig. 1). The abundance of planktonic cells for all bacterial isolates, except *P. fluorescens*, was higher under MRG than under normal gravity on days 1, 2 and 3. Also, *P. fluorescens* and *S. maltophilia* isolate 2 showed higher numbers on day 7 under MRG as compared with normal gravity. Differences in abundance between MRG and normal gravity were greatest for *S. maltophilia* isolate 2. It appeared from the growth curves that there were two stages and that the second stage commenced on approximately day 2 or day 3 under MRG or normal gravity, respectively.

When the bacterial isolates were grown in 0.002% NB, the only significant differences as compared with normal gravity were observed for *Chryseobacterium* sp., which showed a decrease under MRG on day 1, whereas *S. maltophilia* isolate 2 showed an increase under MRG on days 3 and 7 (Fig. 2).

The numbers of cells of *Chryseobacterium* sp. and *S. maltophilia* isolate 1 attached to the steel disks in 0.2% NB were not significantly different under MRG by comparison with normal gravity, regardless of the method of enumeration (Fig. 3). For *P. fluorescens*, cells that stained with SYTO 9 + PI (live + dead) and DAPI were attached to the disks at significantly higher numbers on day 2 under MRG as compared with normal gravity. For *S. maltophilia* isolate 2 (based on live, live + dead and DAPI) numbers of cells that attached to the steel disks was significantly higher under MRG on day 2. In addition, only *Chryseobacterium* sp. grown in either MRG or normal gravity, in 0.2% NB, attached at significantly higher numbers than when grown in 0.002% NB.

Figure 4 shows the attachment of each of the bacterial isolates to steel disks when grown in 0.002% NB. The only significant effect of modeled reduced gravity was observed for *S. maltophilia* isolate 2, which had significantly lower numbers of dead cells attaching to the disks on day 3.

The total number of cells attached to the steel disks may be influenced by the differences in the number of planktonic cells and higher densities of planktonic cells may contribute to increased attachment. To determine

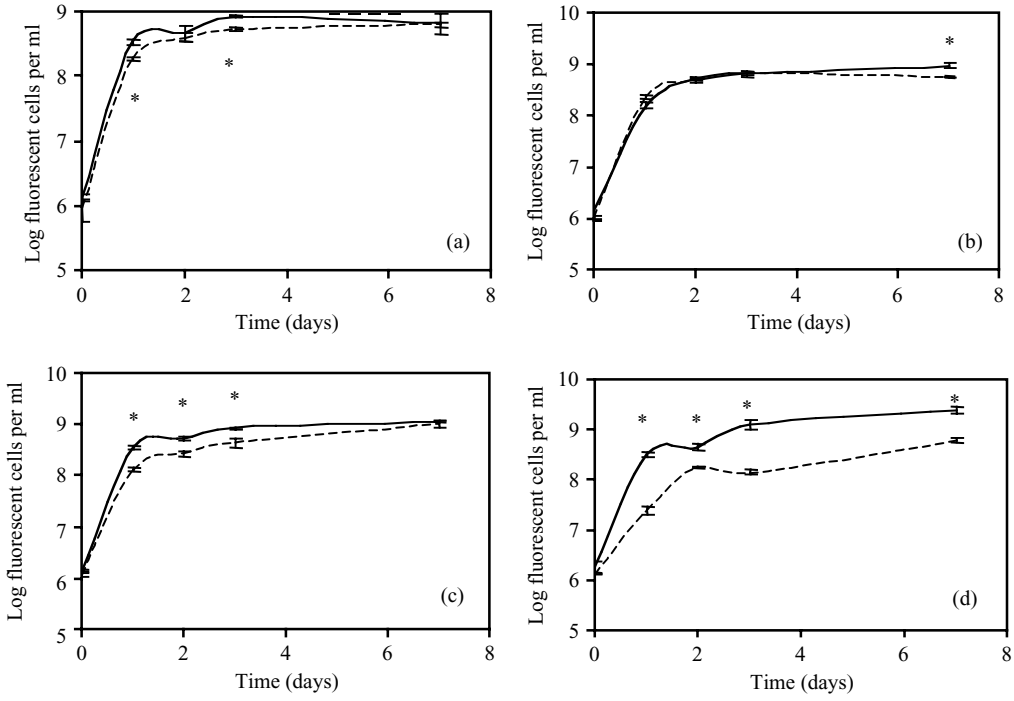

Fig. 1: DAPI counts of planktonic cells of (a) *Chryseobacterium* sp., (b) *P. fluorescens*, (c) *S. maltophilia* isolate 1 and (d) *S. maltophilia* isolate 2, grown in 0.2% (w/v) nutrient broth under modeled reduced gravity (——) and normal gravity (– – –). Significant differences at $P < 0.05$ between reduced and normal gravity are shown with asterisks.

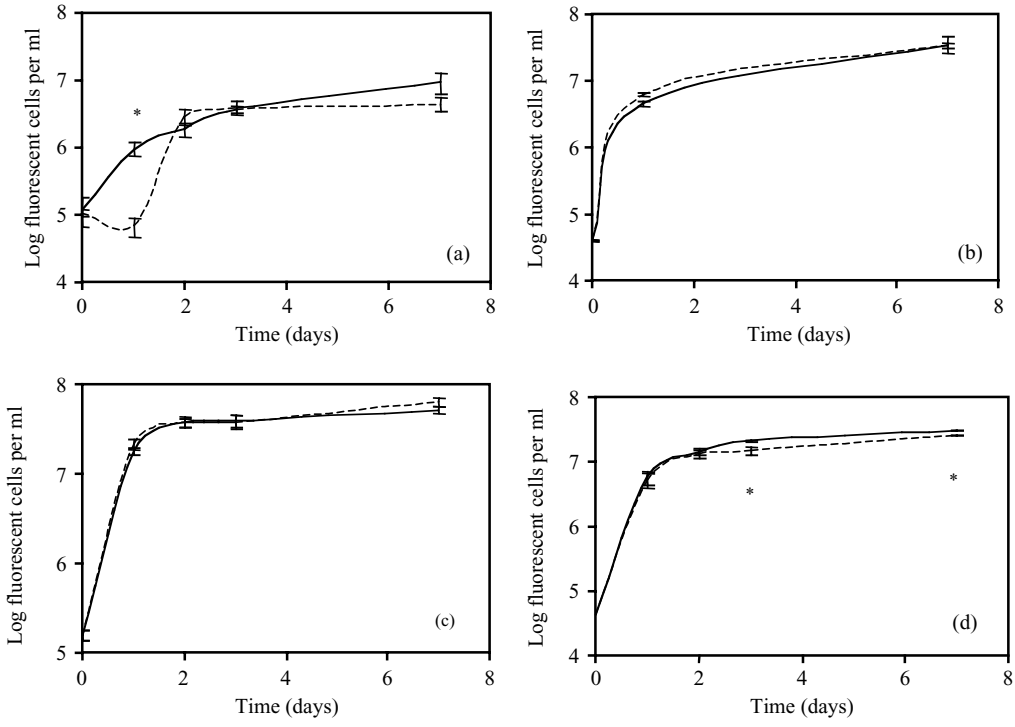

Fig. 2: DAPI counts of planktonic cells of (a) *Chryseobacterium* sp., (b) *P. fluorescens* (note: there are no data for days 2 and 3), (c) *S. maltophilia* isolate 1 and (d) *S. maltophilia* isolate 2, grown in 0.002% (w/v) nutrient broth under modeled reduced gravity (——) and normal gravity (– – –). Significant differences at $P < 0.05$ between reduced and normal gravity are shown with asterisks.

whether other factors might be influencing attachment under the different gravity conditions, it was necessary to account for the differing numbers of planktonic cells between MRG and normal gravity. When differences in planktonic cell abundance are accounted for, only two bacterial isolates grown in 0.2% NB showed significant

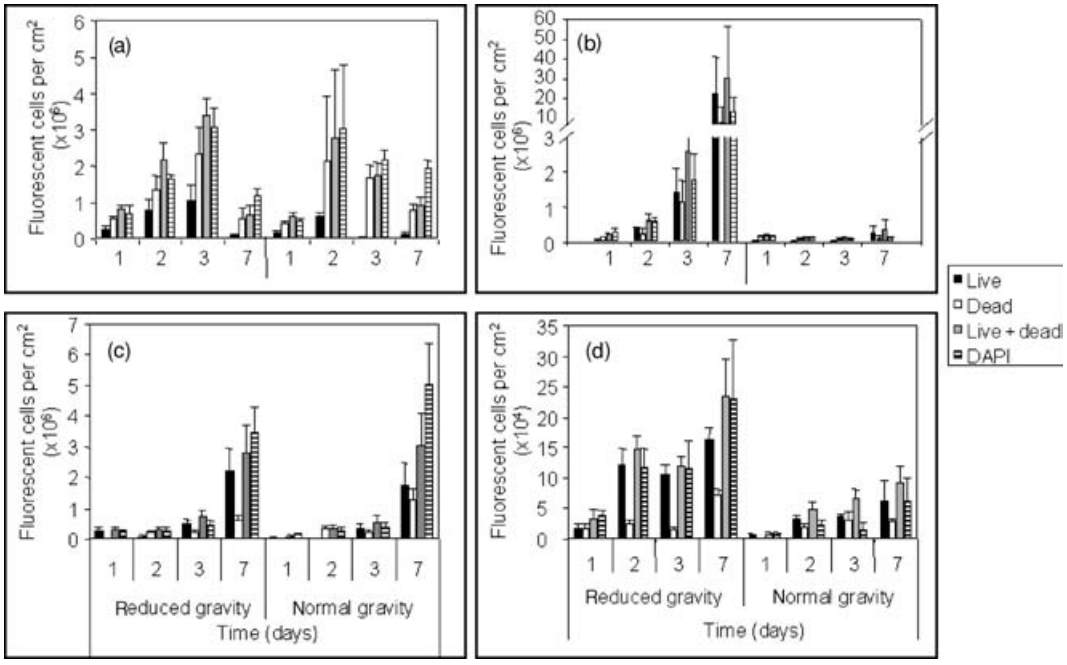

Fig. 3: Cells of (a) *Chryseobacterium* sp., (b) *P. fluorescens*, (c) *S. maltophilia* isolate 1 and (d) *S. maltophilia* isolate 2, attached to stainless steel disks under modeled reduced gravity and normal gravity when grown in 0.2% (w/v) nutrient broth. Vertical lines at the top of the bars represent standard errors.

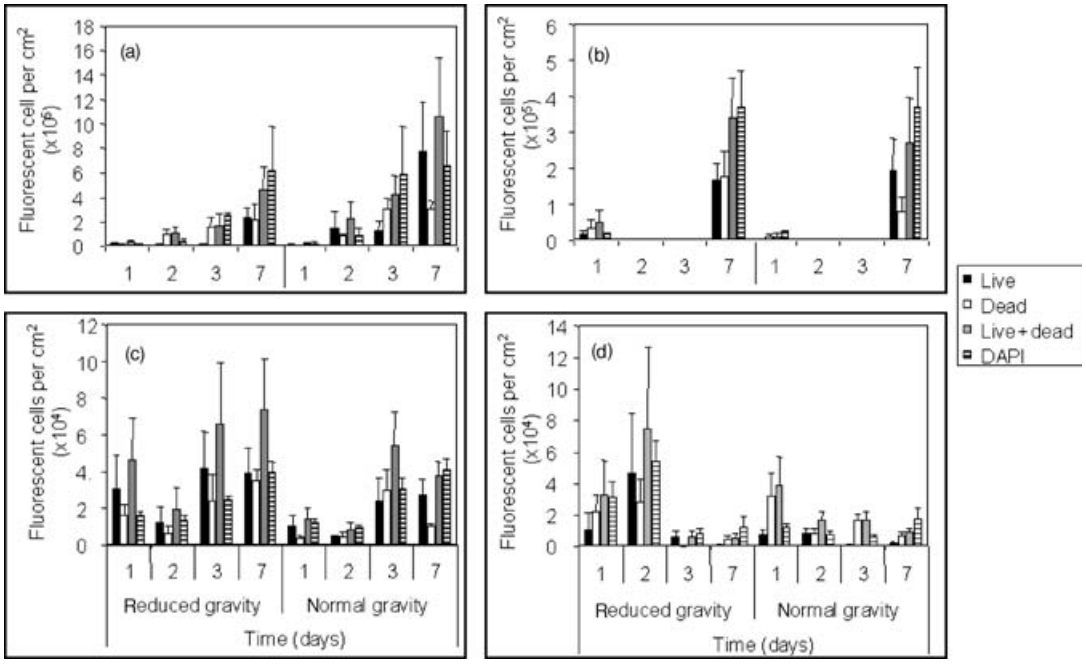

Fig. 4: Cells of (a) *Chryseobacterium* sp., (b) *P. fluorescens*, (c) *S. maltophilia* isolate 1 and (d) *S. maltophilia* isolate 2, attached to stainless steel disks under modeled reduced gravity and normal gravity when grown in 0.002% (w/v) nutrient broth. Vertical lines at the top of the bars represent standard errors.

differences in the fraction of cells attached to the steel disks divided by the total number of bacteria in the STLV (Table 1). This fraction was higher under MRG for *P. fluorescens* on day 2 as compared with the control, whereas it was significantly lower under MRG as compared with the control for *S. maltophilia* isolate 1 on day 1. No significant effects of MRG were observed for attached cells in 0.002% NB (Table 2).

Table 1: Calculated values (values are $\times 10^{-3}$) of attached cells that take into account the number of planktonic cells for bacterial isolates grown in 0.2% nutrient broth in the STLV. For this calculation, the total number of cells attaching to each steel disk was divided by the total number of planktonic cells in a given STLV. Modeled reduced gravity and normal gravity are represented by $<1\,\boldsymbol{g}$ and $1\,\boldsymbol{g}$, respectively. Values in parenthesis are standard errors and values shown in **bold** type are significantly different at $P < 0.05$ between reduced gravity and normal gravity

		Time (days)			
		1	2	3	7
Chryseobacterium sp.	$<1\,\boldsymbol{g}$	1.90 (0.52)	4.35 (1.48)	3.57 (0.56)	2.51 (0.87)
	$1\,\boldsymbol{g}$	2.74 (0.41)	7.46 (4.14)	3.98 (0.67)	3.06 (0.34)
P. fluorescens	$<1\,\boldsymbol{g}$	1.83 (0.56)	**1.16 (0.18)**	2.39 (0.855)	11.8 (5.46)
	$1\,\boldsymbol{g}$	0.71 (0.23)	**0.24 (0.048)**	0.14 (0.02)	0.22 (0.14)
S. maltophilia isolate 1	$<1\,\boldsymbol{g}$	**0.64 (0.14)**	0.44 (0.25)	0.56 (0.12)	3.03 (0.69)
	$1\,\boldsymbol{g}$	**1.23 (0.07)**	0.94 (0.36)	0.76 (0.34)	4.48 (0.62)
S. maltophilia isolate 2	$<1\,\boldsymbol{g}$	0.12 (0.01)	0.28 (0.07)	0.10 (0.02)	0.11 (0.05)
	$1\,\boldsymbol{g}$	0.29 (0.21)	0.17 (0.04)	0.31 (0.07)	0.18 (0.08)

STLV, slow-turning lateral vessel.

Table 2: Calculated values (values are $\times 10^{-3}$) of attached cells that take into account the number of planktonic cells for bacterial isolates grown in 0.002% nutrient broth in the STLV. For this calculation, the total number of cells attaching to each steel disk was divided by the total number of planktonic cells in a given STLV. Modeled reduced gravity and normal gravity are represented by $<1\,\boldsymbol{g}$ and $1\,\boldsymbol{g}$, respectively. Values in parenthesis are standard errors and there were no significant differences between reduced and normal gravity

		Time (days)			
		1	2	3	7
Chryseobacterium sp.	$<1\,\boldsymbol{g}$	20.80 (5.00)	21.90 (4.26)	63.70 (5.43)	1.07 (35.90)
	$1\,\boldsymbol{g}$	2.17 (1.47)	22.70 (12.50)	1.11 (50.10)	61.60 (7.99)
P. fluorescens	$<1\,\boldsymbol{g}$	15.00 (11.40)	ND	ND	11.30 (23.90)
	$1\,\boldsymbol{g}$	3.66 (0.99)	ND	ND	11.40 (3.94)
S. maltophilia isolate 1	$<1\,\boldsymbol{g}$	1.44 (0.59)	0.39 (0.11)	0.68 (0.11)	0.82 (0.23)
	$1\,\boldsymbol{g}$	0.59 (0.09)	0.25 (0.03)	0.95 (0.29)	0.65 (0.06)
S. maltophilia isolate 2	$<1\,\boldsymbol{g}$	5.97 (2.20)	4.15 (1.28)	0.38 (0.18)	0.40 (0.20)
	$1\,\boldsymbol{g}$	2.53 (0.71)	0.57 (0.18)	0.43 (0.16)	0.92 (0.34)

STLV, slow-turning lateral vessel; ND, not determined.

DISCUSSION

Planktonic cell counts of all isolates were significantly higher under modeled reduced gravity as compared with normal gravity in some experiments. This corresponds well with the results of previous studies (Klaus *et al.*, 1997; Kacena *et al.*, 1999a,b; Brown *et al.*, 2002; Baker & Leff, 2004). However, *S. maltophilia* isolate 2 showed a

much greater average difference in cell numbers, 6.1-fold, under modeled reduced gravity as compared with normal gravity. Both *S. maltophilia* isolates are hydrophilic but only isolate 1 has flagella (Song & Leff, 2005). It is likely that bacterial cells that are non-motile remain in a relatively more fixed position as the medium rotates under a modeled reduced-gravity environment. In diluted medium (0.002% NB), the difference in growth under modeled reduced gravity and normal gravity was generally not apparent, except for *S. maltophilia* isolate 2. This indicates that the effect of modeled reduced gravity on bacterial numbers may be lower in dilute medium and, in some instances, may be undetectable.

The numbers of bacteria from a modeled reduced-gravity environment attaching to the steel disks were similar to those found in a previous study done under normal gravity conditions (Hood & Zottola, 1997). It must be noted that cells attaching to the steel disks are under normal gravity conditions because the disks rotate with the STLVs. The number of attached cells of *S. maltophilia* isolate 2 was significantly higher when the cells were grown under modeled reduced gravity as compared with normal gravity. Further calculations that took into account the number of planktonic cells, revealed that the attachment of *S. maltophilia* isolate 2, unlike that of *P. fluorescens*, was probably due to the higher abundance of planktonic cells. One possibility for the increased attachment of *P. fluorescens*, when it was grown under modeled reduced gravity as compared with normal gravity, is the change in the level of expression of different genes involved in adhesion. Previous studies have shown that modeled microgravity induces shifts in the regulation of genes (Wilson *et al.*, 2002) and that hydrophobicity plays an important role in the adhesion of the bacterial cells to stainless steel (Vanhaeke *et al.*, 1990). Cells of different sizes and shapes show different rates of sedimentation (Nagel *et al.*, 1997), and it is also possible this may influence attachment under modeled reduced gravity as compared with normal gravity. In this study, much greater differences were found between modeled reduced gravity and normal gravity for bacteria grown in 0.2% NB as compared with 0.002% NB. This was probably attributable, in part, to the possibility that cells in the 0.2% NB were larger than in the diluted medium because bacterial cell sizes vary with availability of nutrients (e.g. Smith & Prairie, 2004).

When bacteria in this study attach to the steel disks from a modeled reduced-gravity environment, they essentially became subjected again to normal gravity conditions. It is possible that the rate of biofilm development is similar to normal gravity environments. This possibility appears to be reasonable, on the basis of a previous study that showed that the rate of substrate utilization was not significantly different under microgravity as compared with gravity controls, even though the numbers of cells under microgravity was higher (Brown *et al.*, 2002). This suggests that attached cells under modeled reduced gravity may not be limited by resource availability. A comparison of attachment under the high and low nutrient concentrations used in this study revealed that only *Chryseobacterium* sp. showed differences in attachment under the two nutrient conditions.

Numbers of planktonic cells tended to be higher under modeled reduced gravity. This is perhaps because of the lack of sedimentation that occurs under normal gravity, which leads to a more even distribution of cells in the vessels and thus optimizes resource use by the bacterial populations (Klaus *et al.*, 1997). Klaus *et al.* (1997) showed that there was a reduced lag phase, a more rapid growth during the growth phase and higher numbers during the stationary phase. Our results revealed that all of the isolates exhibited small increases in planktonic cell numbers but this increase was much greater for one particular isolate. The results of this study suggest that the attachment of *P. fluorescens* may be affected under a modeled reduced-gravity environment. For other bacteria, although rates of biofilm development may be greater, this is perhaps caused by the increased number of planktonic cells, resulting in more cells that can attach to surfaces under a modeled reduced-gravity environment.

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