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Payload hardware and experimental protocol development to enable future testing of the effect of space microgravity on the resistance to gentamicin of uropathogenic *Escherichia coli* and its σ^s-deficient mutant

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ABSTRACT

Human immune response is compromised and bacteria can become more antibiotic resistant in space microgravity (MG). We report that under low-shear modeled microgravity (LSMMG), stationary-phase uropathogenic *Escherichia coli* (UPEC) become more resistant to gentamicin (Gm), and that this increase is dependent on the presence of σ^s (a transcription regulator encoded by the *rpoS* gene). UPEC causes urinary tract infections (UTIs), reported to afflict astronauts; Gm is a standard treatment, so these findings could impact astronaut health. Because LSMMG findings can differ from MG, we report preparations to examine UPEC's Gm sensitivity during spaceflight using the *E. coli Anti-Microbial Satellite* (*EcAMSat*) as a free-flying "nanosatellite" in low Earth orbit. Within *EcAMSat's* payload, a 48-microwell fluidic card contains and supports study of bacterial cultures at constant temperature; optical absorbance changes in cell suspensions are made at three wavelengths for each microwell and a fluid-delivery system provides growth medium and predefined Gm concentrations. Performance characterization is reported here for spaceflight prototypes of this payload system. Using conventional microtiter plates, we show that Alamar Blue (AB) absorbance changes can assess the Gm effect on *E. coli* viability, permitting telemetric transfer of the spaceflight data to Earth. Laboratory results using payload prototypes are consistent with wellplate and flask findings of differential sensitivity of UPEC and its Δ*rpoS* strain to Gm. if σ^s plays the same role in space MG as in LSMMG and Earth gravity, countermeasures discovered in recent Earth studies (aimed at weakening the UPEC antioxidant defense) to control UPEC infections would prove useful also in space flights. Further, *EcAMSat* results should clarify inconsistencies from previous space experiments on bacterial antibiotic sensitivity and other issues.

1. Introduction

This paper is concerned with the development of a system of hardware and experimental protocols, to be deployed aboard a free-flying "nanosatellite", for determining the effect of space microgravity (MG) on the resistance of uropathogenic *Escherichia coli* [UPEC; strain AMG1 (Wang et al., 2014)] to the antibiotic gentamicin (Gm). This system is termed the *E. coli* Antimicrobial Satellite (*EcAMSat*) payload platform.

UPEC is a causative agent of urinary tract infection (UTI), which has been reported in astronauts (Singh and Matin, 2016), and Gm is standard treatment for this disease. In our previous studies using conventional Earth experimental systems, we have shown that proteins controlled by the sigma factor, σ^s (product of the *rpoS* gene), contribute to the Gm resistance of UPEC; these proteins belong to the antioxidant defense of this bacterium and we have identified several of them. This opens the way for enhancing the effectiveness of Gm against UPEC by devising means (e.g., small-molecule inhibitors) to impair the activity of these proteins (Wang et al., 2014). (σ^s is the master regulator of the general stress response (GSR) in UPEC and several other bacteria; GSR

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is activated under stressful conditions and confers broad resistance on bacteria to diverse antimicrobial agents (Hengge-Aronis, 2002b; Matin, 1991, 2014; Matin et al., 1989)).

We show here (*Results*) that UPEC cultivation in low-shear modeled microgravity (LSMMG) makes it more resistant to Gm compared to its normal gravity-cultivated counterpart and that this enhanced resistance is also dependent on σ^s. This is consistent with previous findings from our and other groups showing that LSMMG cultivation makes UPEC and other bacteria resistant to disinfectant agents, e.g., low pH, high osmolarity, and ethanol (Gao et al., 2001; Lynch et al., 2004). We also showed that the LSMMG-conferred resistance to the disinfectants depends on σ^s (Lynch et al., 2004). Given that the latter regulates the stress-induced GSR mentioned above, the σ^s-dependence of the LSMMG effect suggests that UPEC responds to this condition as a stress.

We are interested in determining whether the UPEC Gm resistance increases also in space MG in a σ^s-dependent manner. While LSMMG may approximate space MG, its findings are not necessarily applicable to the latter. For example, the virulence of *Listeria monocytogenes*, methicillin-resistant *Staphylococcus aureus* (MRSA), and *Enterococcus faecalis* were affected differently by spaceflight compared to LSMMG (Hammond et al., 2013); in *Salmonella enterica* serovar Typhimurium, the virulence genes *sipD* and *sipC* were differently regulated under the two conditions (Wilson et al., 2002).

The *EcAMSat* nanosatellite, scheduled to be launched in 2017, will be used to address the above and related questions. Should the Gm resistance of UPEC increase also in space MG, it would constitute a clear hazard to astronaut health, especially given that human immune response is weakened in space MG (Bascove et al., 2009; Crucian et al., 2009; Mehta et al., 2000; Yi et al., 2016). Further, should it also turn out that the increase in GM resistance in space MG is dependent on σ^s, measures discovered in Earth studies mentioned above, namely impairing the antioxidant proteins (Wang et al., 2014), would be likely to prove useful for increasing Gm effectiveness in treating UPEC infections in space flight. Moreover, indication would be forthcoming that UPEC responds to space MG as a stress.

As in our previous work (Wang et al., 2014; Matin, 2009; Zgurskaya et al., 1997), our focus here is on stationary-phase UPEC. This is because: (a) due, for example, to lack of nutrients or the presence of oxidative stress, this late-growth phase is often experienced by bacteria in the human host (Matin, 2014; Hengge-Aronis, 2002a, Kolter et al., 1993); (b) it is in the stationary phase that bacteria express many of the virulence traits required for disease causation (Sonenshein, 2005; Llorens et al., 2010; Cabeen, 2014; Dalebroux et al., 2010; Mangan et al., 2006; Mouslim and Hughes, 2014; Roop et al., 2003) (an example is UPEC Type I fimbriae, which it uses in bladder colonization (Kau et al., 2005a, b)); and (c) bacteria in this phase are hard to eradicate, because the GSR activated in this phase makes bacteria broadly resistant (Matin, 1991, 2014).

Nanosatellite systems, such as *GeneSat, PharmaSat*, and *O/OREOS*, were developed by NASA to augment the capability for studying microbial behavior in space MG over that offered by the Space Shuttle and the International Space Station (Nicholson et al., 2011; Woellert et al., 2011; Ricco et al., 2011). The nanosatellite platforms do not require crew member participation and have the advantage of permitting experimentation in multiple orbital locations (Nicholson et al., 2011; Woellert et al., 2011; Ricco et al., 2011). The *PharmaSat* platform has previously been used to determine the effect of flight in a low Earth orbit (under conditions that provided $<10^{-3} \times$ Earth gravity) on the sensitivity of the yeast *Saccharomyces cerevisiae* to the antifungal agent voriconazole (Ricco et al., 2011). In developing the *EcAMSat* payload, we built upon the *PharmaSat* system to make it suitable for work with bacteria, and have optimized it for spaceflight implementation of the experiments reported here. We have tested the *EcAMSat* payload system using the same experimental command sequence that will be employed during space flight and show here that it faithfully reproduces the previously reported difference in sensitivity to Gm between UPEC strain AMG1 and its isogenic *rpoS* mutant (Wang et al., 2014).

2. Materials and methods

2.1. LSMMG effect on Gm sensitivity

To determine the effect of cultivation under LSMMG on UPEC, strain AMG1, and its isogenic Δ*rpoS* mutant (Wang et al., 2014), the two strains were cultivated in high-aspect-ratio-vessel (HARV) reactors as described previously (Lynch et al., 2004). Pairs of the reactors were rotated about appropriate axes: vertical for normal gravity ('HARV NG') and horizontal for LSMMG conditions. 50 mL of Luria broth (LB) medium was used in each vessel. Overnight conventional-flask LB cultures were used as inoculum; the starting absorbance at 660 nm (A_{660}) was 0.1, and the HARVs were rotated at 25 rpm. Following 24-h incubation (37 °C), the stationary-phase cells were harvested from the HARVs, re-suspended in M9 salts (referred to from hereon as 'M9') to an A_{660} of 0.4, and mixed with sufficient Gm to give a final concentration of 16 μg/mL. Gm at 16 μg/mL represents the minimum inhibitory concentration (MIC) for resistant (*R*) Gram-negative bacteria. (Gm MICs for intermediate (*I*) and sensitive (*S*) strains are 8 and 4 μg/mL, respectively.)

After 24-h incubation (37 °C) under static conditions, viability was determined by counting colony-forming units (CFU) using LB plates.

2.2. Determination of the suitability of Alamar Blue to assess Gm effect on UPEC viability

To test the effect of space MG in spaceflight experiments, a method for UPEC viability assessment is needed, the results of which can be transmitted from space to Earth via telemetry. The dye Alamar Blue (AB) was used for this purpose in the *PharmaSat* experiment, as it changes color upon reduction (Parra et al., 2009). AB can be reduced by several cellular electron donors (e.g., NAD(P)H, FADH and several cytochromes) and thus its reduction can reflect cell viability. The *PharmaSat* mission involved exponentially growing cells, and it is in this phase that AB is recommended for assessing cell viability (Rampersad, 2012). Our work, however, involved stationary-phase cells and since AB-based measurements can also be affected by factors such as the nature of mutations and test compound or drugs (Rampersad, 2012), it was necessary to determine whether, under the conditions used, AB reduction kinetics could accurately reflect changes in viability of the stationary-phase cells of the two strains by Gm treatment.

Reduction of AB is indicated by a change in its color from dark blue, as measured by red absorbance at 615 nm (A_{615}) to magenta, as measured by green absorbance at 525 nm (A_{525} nm), resulting in concomitant decrease and increase of the absorbance, respectively, at the two wavelengths. Both the reduced and oxidized AB have weak absorbance at 470 nm, and measurement at this wavelength reflects medium turbidity and can thus be used to monitor changes in cell population.

Measured absorbance at 615, 525, and 470 nm only approximate the respective amounts of oxidized AB, reduced AB, and cell-related turbidity. To more accurately determine these parameters, we measured complete visible absorbance spectra of oxidized AB, reduced AB, and a suspension of *E. coli*. We then used the absorbance values at the three measurement wavelengths to calculate "cross terms" that correct for the fact that the absorbance spectrum of (blue) oxidized AB has a shoulder at 525 nm and a tail at 470 nm, that the spectrum of (magenta) reduced AB also has a tail at 470 nm, and that light scattering by the bacteria occurs throughout the visible range, varying with a weak linear wavelength dependence. All graphics and results reported below for quantities of oxidized AB, reduced AB, and cell turbidity have been corrected accordingly.

To determine if the AB-conversion method can be used for assessing