

The effect of simulated microgravity on microbial gene expression

George E. Fox, Richard C. Willson, Duane L. Pierson, T. Madhan Raghaven, and Faithi Karouia

ABSTRACT—The effect of long-term exposure to low-shear modeled microgravity (LSMMG) on microbial gene expression and physiology in *Escherichia coli* is being examined using functional genomics, and molecular techniques. In previous short-term studies, reproducible changes in transcription were seen but no direct responses to changes in the gravity vector were identified. Instead, absence of shear and a randomized gravity vector appeared to cause local extra-cellular environmental changes, which elicited reproducible cellular responses. Current efforts are focused on assessing the extent to which the response changes over the long term as a result of evolutionary adaptation. To this end, a cell line has now been cultured in the LSMMG environment for 1,000 generations and is currently being studied in detail. Expression studies reveal that 357 genes are either up- or down-regulated relative to the unevolved strain grown under the same conditions in the same media. This clearly establishes that a long-term adaptation occurs. Ongoing efforts are focused on understanding the molecular basis of the adaptation and how it is advantageous to the evolved cell lineage.

INTRODUCTION

Bacteria are capable of living in and adapting to a far broader range of environmental conditions than are normally encountered in the usual laboratory environments. Even with full knowledge of an organism's gene content, it is currently impossible to predict how expression patterns will change in different situations. Thus, usual laboratory growth conditions may not elicit key aspects of an organism's potential response, and therefore such studies may conceal behaviors that in a different environment may contribute to undesirable phenomena such as pathogenesis. Examples include the low-shear, low-turbulence environment present *in utero*, at the brush border microvilli of epithelial cells, and in other medically important host environments.¹ Similarly, the space environment characterized by microgravity and high background radiation produces a low-shear environment that likely inhibits the movement of microorganisms away from nutrient-depleted surroundings contaminated by waste products.²

In order to adapt to life in a low-shear world, bacteria likely express different combinations of genes than they do in more usual laboratory environments and may ultimately make evolutionary adaptations as well. Thus, a particular bacterium may exhibit properties such as antibiotic resistance, biofilm formation, or virulence not generally associated with it. It is not certain which organisms may be problematic. For example, a recent study³ showed that *Salmonella enterica* serovar Typhimurium grown under low-shear modeled microgravity (LSMMG) appeared to have increased virulence potential in a murine model system. A follow-up study⁴ revealed that a significant number of the genes are transcriptionally regulated in response to LSMMG. Thus, it is essential to better understand both the long- and short-term effects of microgravity on bacterial behavior.

METHODOLOGY

Wild type *E. coli* MG1655 (CGSC7740) is grown aerobically at 37°C, in rich (LB) medium under LSMMG and in a normal gravity vector control environment. In order to conduct long-term studies, a sterilization procedure was developed in which two reactors were alternately used. The reactor not in use was rinsed with a disinfectant solution and exposed to UV light. This protocol has allowed cultures to be maintained for more than 1,000 generations. Total RNA was harvested from the mid-log phase of growth. The SuperScript™ Double-Stranded cDNA Synthesis System (Invitrogen Corporation) was used to generate cDNA from the RNA samples for gene expression studies on microarrays. Double-stranded cDNA was random-prime labeled with Cy3-nonamers and hybridized with microarrays to analyze gene expression.

High-density monoplex microarrays (Roche NimbleGen Systems, Inc., Madison, WI) were used that were based on the sequence and annotation data for *Escherichia coli* K12 NC_000913. The arrays consisted of 4,254 open reading frames (ORFs) of the compiled genome. Each ORF was represented on the glass slide by 18 unique probes of 60-mer oligonucleotides. Each unique oligonucleotide was repeated five times on the array for a total of ~385,000 features. The microarrays were scanned using an Axon GenePix 4000B scanner (Molecular Devices Corp., Union City, CA) at 5 μm resolution. The data were extracted from the raw images using NimbleScan™ software (Roche NimbleGen). In the one-generation control sample, *E. coli* cells were grown in the LSMMG environment until they reached mid-log phase. The experimental sample was exposed to the same environment for over 1,000 generations. In each case there were two biological replicates. A total of four microarrays were used. The signal

intensities for each feature were generated using the robust multi-array average (RMA) algorithm.^{5,6} The data were processed based on quantile normalization.⁷ This normalization method aims to make the distribution of intensities for each array in a set of arrays the same by assuming that a quantile-quantile plot of two data vectors with the same distribution will have a straight diagonal line. A *P* value for each ORF was calculated by two-tailed Welch's unpaired *t* test comparison of the two microarray replicates for each sample.

Relative changes in signal intensity of an ORF between samples were calculated as the following ratio: average 1000G signal intensity/average 1G signal intensity. Only ORFs with relative changes of at least 2 and a *P* value less than or equal to 0.05 were considered significant. The Colibri WWW Server v3.1 <<http://genolist.pasteur.fr/Colibri/genome.cgi>> was used to determine gene nomenclature, location, and orientation in the *E. coli* genome as well as possible co-transcription with other expressed genes. EcoCyc (Institute for Genomic Research, University of California; San Diego, CA, <<http://ecocyc.org/>>) and EcoSearch (University of Miami School of Medicine; Miami, FL, <<http://bmb.med.miami.edu/search.php>>) were also used in determining gene name synonyms and gene product function.

EQUIPMENT/SPECIAL TECHNOLOGY

The project takes advantage of the high-aspect rotating vessel (HARV) bioreactor originally developed by NASA scientists⁸ to minimize fluid motion for tissue culture differentiation while maintaining culture aeration through a gas-permeable membrane. The HARV's rotation also has the effect of randomizing the gravity vector by rotating in the plane of gravity, producing the LSMMG environment. To obtain this environment, the HARV device is rotated at a speed sufficient to maintain cell suspension in the media and completely filled, thereby preventing gas bubbles from causing solution turbulence (i.e., shear). The HARV apparatus approximates the physiological and transcriptional changes occurring in space flight due to microgravity, while allowing Earth-based culturing. Used in conjunction with commercially available functional genomics technology (Panorama Gene Arrays, Sigma-Genosys), the HARV makes it possible to study microbial gene expression on a genome-wide basis under LSMMG.

RESULTS

The analysis identified 237 up-regulated and 120 down-regulated genes in the long-term exposure to LSMMG compared to the short-term exposure to LSMMG during the mid-log phase of growth in rich medium. These genes were catego-

Table 1. Functional clustering of genes significantly up- or down-regulated

	Up-regulated Genes	Down-regulated Genes
Pilus/Lipopolysaccharide/Biofilm	19	1
Mobility	0	13
Cell Protection/Adaptation	2	3
Prophage/Phage/Transposon	18	12
Regulation/Chaperon	29	23
Metabolism	32	28
Transport	61	10
Membrane	20	4
DNA Replication/Recombination and Repair/RNA Modification	3	10
Putative of Conserved Protein/ of Unknown Function	19	7
ORF of Hypothetical Protein	34	9

rized, as shown in Table 1. Eighty-one of the up-regulated genes are known to be directly or indirectly associated with biofilm formation. The remaining up-regulated genes seem to be involved in a response that triggers: (1) gene members of the Type II secretion or secretin complex known as the general secretory pathway (GSP), for the export of proteins across the outer membranes of Gram-negative bacteria (related to virulence factors); and (2) gene members of key elements of the multidrug efflux system that confer resistance to a multitude of antimicrobial agents and antibiotics.

Such general transcriptional expression implies that the *E. coli* cells may be predisposed to forming aggregates either in solution or on the surface of the HARV as well as increased resistance to antibiotics. The analysis also revealed several transcriptional regulators that were differently expressed under the experimental conditions. However, none of these was apparently associated with the genes that were highly up-regulated (fimbrial complex). Therefore, long-term exposure to the LSMMG may have significant unexpected value in future work aimed at fully understanding the regulation of the *fim* operon. A recent study with the close *E. coli* relative *Salmonella typhimurium* suggested that Hfq played a role in spaceflight and LSMMG response as compared to normal gravity as a global regulator.⁹ However, in the case of *E. coli*, the level of expression of Hfq was not significantly changed, and the expression level of only a limited number of genes associated with Hfq changed to a significant extent.

DISCUSSION

Despite the difference in expression patterns relative to the non-adapted control strain, no significant changes in growth kinetics were observed. So what adaptive advantage has been achieved? The increased elevated expression of genes associated with antibiotic resistance suggests an attempt by the cells to circumvent the cleaning process. The increase in expression

of genes associated with biofilm formation is consistent with the likely advantage of overcoming the low shear environment that would make it difficult for a cell to remove itself from a microenvironment that had become nutrient-poor. At this point, however, we have not visualized biofilm formation in the HARV. Thus, further investigation is required and could involve analyzing cellular aggregation and clumping by scanning electron microscopy. Overall, it remains unclear what are the real cellular effects of long-term exposure to LSMMG, as compared to the short term. One way to address this and assess small differences in growth kinetics could be achieved by using a more sensitive fitness assay. Finally, since only one time point was examined, it remains to determine how rapidly adaptation occurs and how reproducible it is. More generally, a large portion of the up-regulated genes and down-regulated genes of known function are present in or involved with regulation of the cellular envelope. This suggests that the cell envelope is superlative in sensing changes in its local environment and able to rapidly respond to the changes in a multifaceted way.

CONCLUSIONS

Substantial differences in gene expression were seen between the 1000G strain and the 1G control, providing significant evidence that long-term adaptation is occurring. In the future, re-sequencing of the genome is planned in order to determine how much genetic change has actually occurred. Having lived on the Earth for billions of years, bacteria have not typically encountered microgravity. Hence it would seem unlikely that genes governing a direct response to variations in gravity would have evolved. With specific reference to the LSMMG environment then, it would be anticipated that low shear is more important in the bacterial transcriptional response than a direct effect of the randomized gravity vector. Experiments to date have confirmed this conclusion. In addition, our studies have reinforced the hypothesis that the cell envelope is superlative in sensing changes in its local environment and able to rapidly respond to changes in a multifaceted way. The failure of Hfq and associated genes to be up-regulated, as occurred in *Salmonella*, further reinforces our earlier observation that even closely related organisms respond to the low-shear environment differently. This is a frustrating but important conclusion for those hoping to ascertain what the effect of exposure to low shear or the space environment will be for microorganisms in general.

ACKNOWLEDGMENTS

We would like to thank Yuriy Fofanov, Ph.D., in the Department of Computer Science at the University of Houston, for his advice and assistance in performing statistical analyses on the microarray data.

REFERENCES

1. Stock, U.A. and Vacanti, J.P. Cardiovascular physiology during fetal development and implications for tissue engineering. *Tissue Eng.* **7**, 1-7 (2001).
2. Jessup, J.M. and Pellis, N.R. NASA biotechnology: Cell science in microgravity. *In Vitro Cell Dev. Biol. Anim.* **37**, 61-63 (2001).
3. Nickerson, C.A., Ott, C.M., Mister, S.J., Morrow, B.J., Burns-Kelihier, L., and Pierson, D.L. Microgravity as a novel environmental signal affecting *Salmonella enterica* serovar Typhimurium virulence. *Infect. Immun.* **68**, 3147-3152 (2000).
4. Wilson, J.W., Ramamurthy, R., Porwollik, S., McClelland, M., Hammond, T., Allen, P., Ott, C.M., Pierson, D.L., and Nickerson, C.A. Microarray analysis identifies *Salmonella* genes belonging to the low-shear modeled microgravity regulon. *Proceedings of the Natl. Acad. Sci. USA.* **99**, 13807-13812 (2002).
5. Irizarry, R.A., Bolstad, B.M., Collin, F., Cope, L.M., Hobbs, B., and Speed, T.P. Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res.* **3**, e15 (2003).
6. Irizarry, R.A., Hobbs, B., Collin, F., Beazer-Barclay, Y.D., Antonellis, K.J., Scherf, U., and Speed, T.P. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* **4**, 249-264 (2003).
7. Bolstad, B.M., Irizarry, R.A., Astrand, M., and Speed, T.P. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* **19**, 185-193 (2003).
8. Prewett, T.L., Goodwin T.J., and Spaulding, G.F. Three-dimensional modeling of T-24 human bladder carcinoma cell line: A new simulated microgravity culture vessel. *J. Tiss. Cult. Methods* **15**, 29-36 (1993).
9. Wilson, J.W., Ott, C.M., Höner zu Bentrup, K., Ramamurthy, R., Quick, L., Porwollik, S., Cheng, P., McClelland, M., Tsaprailis, G., Radabaugh, T., Hunt, A., Fernandez, D., Richter, E., Shah, M., Kilcoyne, M., Joshi, L., Nelman-Gonzalez, M., Hing, S., Parra, M., Dumars, P., Norwood, K., Bober, R., Devich, J., Ruggles, A., Goulart, C., Rupert, M., Stodieck, L., Stafford, P., Catella, L., Schurr, M.J., Buchanan, K., Morici, L., McCracken, J., Allen, P., Baker-Coleman, C., Hammond, T., Vogel, J., Nelson, R., Pierson, D.L., Stefanyshyn-Piper, H.M., and Nickerson, C.A. Space flight alters bacterial gene expression and virulence and reveals a role for global regulator Hfq. *Proceedings of the Natl. Acad. Sci. USA* **104**, 16299-16304 (2007).