Chapter 14
Spaceflight and Spaceflight Analogue Induced Responses in Gram Positive Bacteria

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Historical Landmarks

1968—Early experiments aboard Gemini and Apollo missions investigate the survival of *Bacillus subtilis* when exposed to the space environment [1].

1982—Experiments aboard the Salyut 7 space station investigating antibiotic sensitivity of *Staphylococcus aureus* indicated the minimum inhibitory concentration when exposed to oxacillin, chloramphenicol, and erythromycin was increased in response to spaceflight culture [2].

1986—*Bacillus subtilis* cultured in the European Space Agency Biorack facility displayed shorter lag growth phase and increases in the rate of cell division and biomass [3].

1999—Studies comparing the response of *B. subtilis* growth on a semi-solid agar and in liquid medium indicate that spaceflight induced alterations in growth profiles and final cell concentrations are the result of fluid dynamics or extracellular transport, rather than a cellular response to gravity [4].

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(continued)

2011—*Staphylococcus aureus* cultured in the Rotating Wall Vessel had increased extracellular polymeric substance production, decreased resistance to whole human blood, and decreased carotenoid production compared to control cultures [5].

14.1　Introduction

Over the past 12 years, the interest in microbial responses to spaceflight culture has dramatically increased as a result of (a) the utilization of the NASA designed rotating wall vessel bioreactor as a spaceflight culture analogue for microorganisms [6–8], (b) advances in and availability of molecular microbiological techniques, and (c) a clear association of spaceflight culture with alterations in virulence and virulence characteristics [9–11]. Many of these studies have focused on Gram negative pathogens, such as *Salmonella enterica* serovar Typhimurium [9, 10, 12], *Pseudomonas aeruginosa* [11, 13, 14], and *Escherichia coli* [15]. However, recent studies investigating the response of Gram positive organisms have provided intriguing insight into the similarities of the responses among dramatically different species and how evolutionarily conserved these responses may be.

The potential alterations in the disease-associated characteristics of Gram positive organisms during spaceflight missions are of great importance for future human exploration efforts. Gram positive organisms, especially *Staphylococcus* species, are the most prevalent species isolated from the air and surfaces of spacecraft vehicles [16, 17]. The frequent isolation of the opportunistic pathogen, *Staphylococcus aureus* [17, 18], from the environment of the International Space Station (ISS) is not unexpected, as 30–50 % of healthy adults on Earth are colonized with this organism [19]. Passage of these organisms between crewmembers is common as demonstrated by a genetic comparison of *S. aureus* strains isolated from crewmembers aboard the Mir space station [20]. Microbial monitoring also indicated the presence of *Streptococcus* species aboard the Russian space station Mir [21] and from air samples collected during Space Shuttle missions [22]. While *S. pneumoniae* has not been isolated from spacecraft or from a crewmember after flight, this opportunistic bacterium has been isolated after nasopharyngeal sampling from a shuttle crewmember [23] immediately before flight. As potential pathogens, such as *S. aureus* and *S. pneumoniae*, are likely to be carried as part of the normal flora of a crew and may exploit a declining immune system, understanding the mechanisms behind the disease causing potential of Gram positive organisms has tremendous implications for the spaceflight crew.
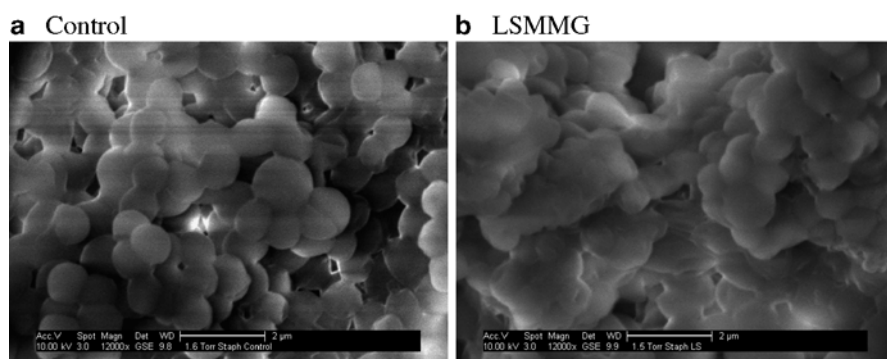
14.2　*Staphylococcus aureus*

S. aureus is a Gram-positive, opportunistic pathogen commonly found on humans and in the environment. This ubiquitous nature is reflected in the common isolation of S. aureus from the environment of the space shuttle, Mir space

station, and ISS [16, 17]. Accordingly, it is important to understand how *S. aureus* responds to the microgravity environment of space and the possible consequences associated with crew health.

In a collaborative effort in 1982, French and Russian crew carried by the Soyuz-T docked with the Salyut 7 space station to carry out a series of biomedical experiments. As part of the Cytos 2 experiment, *S. aureus* was cultured in-flight and assessed for alterations in the minimal inhibitory concentrations (MIC) of oxacillin, chloramphenicol, and erythromycin as compared to ground controls [2]. It was determined that the MIC of all three antibiotics was slightly higher and that the increase in MIC was accompanied by a thickening of the cell wall of spaceflight-cultured *S. aureus* [2]. While this study describes some specific effects of *S. aureus* grown in the microgravity environment of space, it provides only limited characterization of the organism.

The response of several *S. aureus* strains to LSMMG (Low Shear Modeled Microgravity) has been documented by taking advantage of the RWV (Rotaing Wall Vessel) bioreactor [5, 24–27]. The evaluation of *S. aureus* N315 revealed the formation of visible bacterial aggregates in the fluid phase of the LSMMG culture [5]. Analysis of the aggregates by environmental scanning electron microscopy (ESEM) indicated that the LSMMG cultures produced significantly higher amounts of an extracellular polymeric substance (EPS) that enveloped the LSMMG-cultured cells as compared to controls (Fig. 14.1) [5]. When assessed for alterations in antibiotic resistance, the LSMMG-induced aggregates were 1.72-fold more resistant to ciprofloxacin as compared to control-cultured bacteria [5]. The encasement of the cells by an EPS, which confers increased antibiotic resistance, is consistent with characteristics of surface-attached bacterial biofilms [28]. Interestingly, the heavy EPS was in response to LSMMG-culture conditions rather than a more traditional attachment to a solid surface [5].

Fig. 14.1 ESEM images of control- and LSMMG-cultured *S. aureus* N315. Control-cultured *S. aureus* at 10,000× (**a**) magnification demonstrated that individual staphylococcal cells were clearly visible. *S. aureus* cultured under LSMMG conditions at 10,000× (**b**) confirmed that the cells were much less visible and completely encased in an EPS matrix [5]. Copyright © American Society for Microbiology, Applied and Environmental Microbiology, 77(18):6368–6378, 2011

Growth profiles of *S. aureus* in LSMMG-culture differ among strains. Rosado et al. reported that three clinical isolates (designated RF1, RF6, and RF11) displayed comparable growth profiles in LSMMG and control conditions [25]. In contrast, Castro et al. reported LSMMG culture resulted in a 2.9-fold and 5.6-fold lower total cell concentration for N315 and 8325, respectively, as compared to controls [5]. An increased final cell density has also been reported for *S. aureus* 25923; however, the increase was not noted until deep into stationary growth phase after 40 h of culture in the RWV [27]. Therefore, it is possible that the increase noted by the investigators may not be solely based on growth characteristics in LSMMG culture, but instead may be based on the enhanced survival characteristics associated with changes in nutrient depletion and/or waste build-up. Interestingly, the induction of EPS from *S. aureus* cultures is also dependent on the specific strain [5].

While alterations in *S. aureus* characteristics, such as EPS production and growth profiles, in response to LSMMG culture are strain dependent, common phenotypic changes have been reported in multiple strains in response to this environment. For example, a characteristic of most *S. aureus* strains is their golden yellow color arising from the production of the primary carotenoid pigment, staphyloxanthin, expressed during the stationary phase of growth. Visual inspection of LSMMG-cultured *S. aureus* N315 revealed a decrease in the pigmentation of bacterial pellets (Fig. 14.2a) [5]. Extraction, measurement, and comparison of the carotenoids from LSMMG-cultured *S. aureus* N315 to control cultures and unpigmented *S. aureus* 8325 quanti-
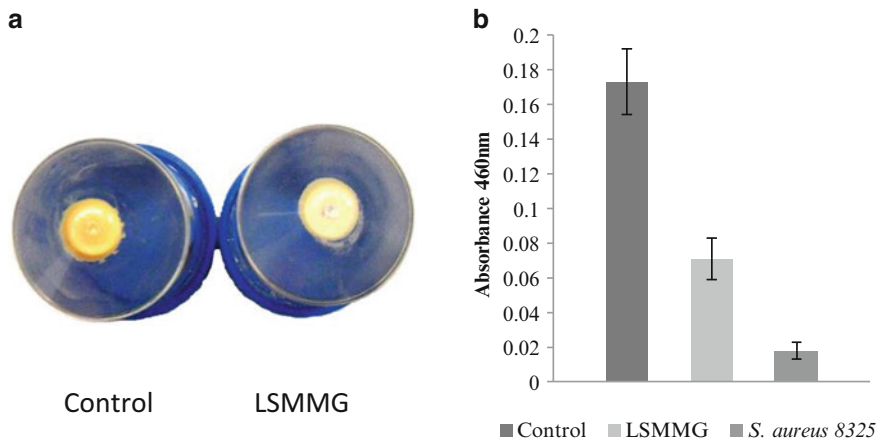
Fig. 14.2 Decreased carotenoid production of *Staphylococcus aureus* N315 in response to LSMMG culture. (**a**) Pellets of control- and LSMMG-cultured *S. aureus* revealing a visual difference in pigmentation of the cells. (**b**) The quantitative difference in pigmentation between control- and LSMMG-cultured *S. aureus* determined by carotenoid extraction and measurement spectrophotometrically at 460 nm. There was a significant reduction in the absorbance of low-shear-cultured bacteria as compared to the control (*, $P < 0.0001$). *S. aureus* 8325, which does not produce carotenoids, was used as a negative control for comparison [5]. Copyright © American Society for Microbiology, Applied and Environmental Microbiology, 77(18):6368–6378, 2011

fied the significant decrease in pigmentation (Fig. 14.2b) [5]. Rosado et al. also reported a reduction in *S. aureus* carotenoid production for the clinical strains RF1, RF6, and RF11 in response to LSMMG culture [25]. Collectively, all pigmented *S. aureus* strains that have been assessed for alterations in carotenoid expression in response to LSMMG have displayed a reduction in carotenoid production, indicating a common response among strains to this environmental parameter [5, 25].

The LSMMG environment has been shown to predispose certain Gram negative bacteria with an increased ability to withstand environmental stressors such as high heat and acidic conditions [29]. As the antioxidant properties of carotenoids serve as a shield protecting *S. aureus* from the toxic effects of reactive oxygen species associated with host immunity [30], and as carotenoid production is impacted by the LSMMG environment, the susceptibility of *S. aureus* to oxidative stress was assessed by Castro et al. [5]. After 60 min of exposure to oxidative stress, 50 % of the LSMMG-cultured *S. aureus* had succumbed to damage, whereas the control culture survival rates did not fall below 90 % [5]. Interestingly, by removing the LSMMG- and control-cultured bacteria from the bioreactor vessels and allowing them to sit statically for a period of time and then repeating the oxidative stress assay, it was found that, at a time greater than 1 h but less than 1.5 h, there was no longer a significant difference between the percent survival of LSMMG- and control-cultured *S. aureus* [5]. Based on this data, Castro et al. estimated the half-life of the observed oxidative sensitivity to be 68.3 ± 1.3 min. As opposed to findings with certain Gram negative organisms, neither *S. aureus* N315 nor 8325 cultured in LSMMG revealed a difference in survival to thermal or acid stress.

S. aureus is associated with bacterial sepsis, and thus its ability to survive in the blood stream impacts its dissemination throughout the body [19]. As a result, the vast majority of *S. aureus* strains have an array of mechanisms to procure nutrients and avoid host immunity upon entering the blood stream [19, 31]. To determine if LSMMG culture impacted the ability of *S. aureus* to survive in human blood, the hemolytic ability of *S. aureus* strains RF1, RF6, and RF11 were assessed [24]. All strains demonstrated a reduction in the ability to lyse sheep or rabbit erythrocytes, with the hemolytic capability of RF6 being almost absent in response to LSMMG-culture conditions. In a separate study, upon challenge with freshly drawn human whole blood, LSMMG-cultured *S. aureus* N315 was approximately 30 % more susceptible to being killed as compared to control cultures [5]. Collectively, these studies demonstrate that the survival of *S. aureus* upon interactions with blood may be impaired immediately following growth in the LSMMG environment.

Analyses of the gene expression of *S. aureus* in LSMMG culture have also provided advances in our understanding of the mechanism(s) behind the microbial response in this environment. Depending upon the strain, the differential regulation of 4–25 *S. aureus* genes has been reported in response to the LSMMG environment [5, 25]. A common change in strains RF1, RF6, and RF11 was the down-regulation of VraX, which is proposed to be involved in the cell wall stress stimulon of *S. aureus* [25]. However, upon creating a *vraX* deletion mutation, no differences were noted in growth, viability, the MIC of multiple antibiotics, pigmentation, hemolytic ability, or the differential expression of any genes when compared to the wild-type

[25]. Examination of the *S. aureus* N315 genes that were up-regulated after LSMMG culture suggested an altered metabolic profile, as many of the protein products of the genes were associated with a fermentative metabolism [5]. Moreover, alignment of the LSMMG-responsive genes revealed the conserved consensus sequences for regulatory proteins Rex and SigB [5]. The expression of these genes, in addition to *hfq,* which has been directly associated with the molecular mechanism governing the LSMMG response in both *S.* Typhimurium [9] and *P. aeruginosa* [14] (see Chaps. 11 and 12), was assessed with quantitative real-time PCR. In stationary phase, no changes in expression levels of Rex or SigB were noted; however, a 2.68-fold decrease in *hfq* expression was observed [5]. While the contribution of Hfq to the molecular regulation of *S. aureus* is unclear [32, 33]; Liu et al. speculated that Hfq was a global regulator in *S. aureus* based upon their investigations of an *hfq* mutant [32]. Interestingly, a side-by-side comparison of the *S. aureus* LSMMG-cultured microarray data from Castro et al. [5] with the work by Liu et al. revealed that 7 of the 17 LSMMG-up-regulated genes were also up-regulated in response to an *hfq* mutation. Additionally, 9 of the 17 LSMMG-induced genes were found by Liu et al. to bind Hfq [32]. Moreover, the work by Liu et al. also reported that the mutation of *hfq* impacted pigment production [32]. Taken together, the decreased expression level of *hfq* in response to LSMMG culture, combined with the significant correlations of this work with that of Liu et al., strongly suggests that Hfq is involved in the LSMMG response of *S. aureus*. More importantly, this is the first description associating an Hfq response to the LSMMG environment in a Gram-positive bacterium [5]. The correlation of Hfq to the LSMMG response of *S. aureus*, in addition to the previously documented responses in Gram-negatives, strongly suggests that the ability to sense and respond to mechanical stimuli is evolutionarily conserved among structurally diverse prokaryotes.

The potentially evolutionarily conserved responses of *S. aureus* to LSMMG culture share common mechanistic characteristics with Gram negative organisms, such as the involvement of Hfq; however, a fundamental difference in how these organisms alter virulence characteristics exists. This difference consistently appears to be based on the benefit toward each microorganism's proliferation and perseverance capabilities in this environment. Contrary to previous reports of enhanced virulence and/or virulence properties of *S.* Typhimurium [12] and *P. aeruginosa* [13], *S. aureus* appears to favor a phenotype consistent with colonization, in which it forms a biofilm and down-regulates virulence characteristics [5, 25]. Collectively, these comparisons may afford a unique opportunity to examine the role of environmental parameters serving as cues directing the balance between infection and colonization by *S. aureus* during the initial host–pathogen interaction.

14.3 *Streptococcus pnuemoniae*

***Streptococcus* is non-motile, non-spore forming catalase-negative, Gram positive bacteria commonly arranged in pairs or chains**. S. *pneumoniae* (pneumococcus) grows commonly in chains or pairs, is optochin sensitive, and is alpha

hemolytic on blood agar plates. *S. pneumoniae* resides primarily in the nasopharynx and is capable of causing a diverse spectrum of disease ranging from otitis media to bacteremia and meningitis [34–36]. It is the leading cause of community-acquired pneumonia, and a principal cause of meningitis and otitis media [36–40]. Normally an opportunistic pathogen, *S. pneumoniae* affects mainly the very young, the elderly, and the immunocompromised. Carriage rates in healthy individuals vary from 5 to 70 % depending on age, environment, and season (*pneumoniae*). Estimates are that 5–10 % of adults without children are colonized with *S. pneumoniae*. At military installations, *S. pneumoniae* carriage rates can be as high as 50–60 % [41]. *S. pneumoniae* can be a commensal inhabitant of the nasopharyx in humans [41, 42], an opportunistic pathogen in individuals with impaired immune systems, and a mediator of serious disease which can be easily spread via aerosols. In the USA, pneumococcal disease is responsible for an about 3000–6000 cases of meningitis, more than 50,000 cases of bacteremia, estimated 175,000 hospitalizations for pneumococcal pneumonia, and 5,000,000 cases of otitis media annually [41]. *S. pneumoniae* is transmitted by aerosols, and is carried in the upper respiratory tract by healthy individuals [41].

The response of *S. pneumoniae* to LSMMG has been extensively examined [43, 44]. In these experiments, controls with RWVs with an axis parallel to the gravity vector, which were rotating or static were compared to LSMMG conditions (axis perpendicular to the gravity vector). This allowed examination of the effect of modeled microgravity on *S. pneumoniae* gene expression as well as the definition of the contribution of rotation in RWV controls as compared to the static controls. Bacterial growth under LSMMG or static or rotating controls showed no differences in the kinetics of growth or density during logarithmic growth [44]. These studies were performed in THY broth; it is not known if minimal or defined media would yield different results as reported for other bacterial species [10].

The effect of LSMMG on *S. pneumoniae* global gene expression was examined. Microarray analysis comparing RWV static controls and LSMMG conditions revealed 101 genes whose expression was altered [44]. These genes represented a broad range of functional groups including those involved in adhesion, cell envelope, cofactor biosynthesis, iron acquisition, metabolism, proteases, stress response genes, toxin production, transcriptional regulation, transporters, and those in other or unknown groups [44]. For static controls versus $1 \times g$ conditions, 63 genes were up-regulated and 38 were down-regulated [44]. This included genes whose encoded proteins were involved those involved in adherence, proteases, stress proteins, and transport proteins among others [44]. Interestingly, 46 of these same genes were also shown to be differentially expressed in the rotating control versus $1 \times g$ [44]. For these experiments, 4 genes were observed to be up-regulated and 42 down-regulated [44]. Shear forces likely explain the changes seen in the 43 genes altered in LSMMG versus either of the controls. Additionally, six genes (including 2 affecting transporter functions and 2 that are regulatory), were shown to be differentially expressed between the static and the rotating control and LSMMG conditions [44]. These observed changes are likely a specific effect of RWV rotation on gene expression. A comparison of static versus rotating RWV controls has not been extensively

reported for other bacterial species but could reveal a conserved repertoire of genes and subsequent properties that arise from the rotational component of RWVs.

Hierarchical clustering comparing the static controls and $1 \times g$ identified a group of 19 genes that had similar expression patterns with 15 showing similar expression differences using four different analysis methods (GenePix Pro 6.0, Spotfire DecisionSite 7.3, Significance Analysis of Microarray, and ANOVA) [44]. Overall, adaptation to LSMMG altered gene expression that could help explain virulence differences observed after spaceflight [44]. Additionally, for *S. pneumoniae*, comparisons of static controls to LSMMG and $1 \times g$ conditions have been found to be exceptionally relevant and should be rigorously evaluated in future experiments [44].

More recently, *S. pneumoniae* was cultured on the ISS (STS-118) and Space Shuttle mid-deck (STS-129) in the SPEGIS (**S**treptococcus **E**xpression of **G**enes **I**n **S**pace) flight experiment (Unpublished Data). The transcriptome of these cultures was compared to ground controls and that obtained for LSMMG cultures. Principal components analysis (Gene Spring after Lowess normalization) also establishes the similarity and distinct qualities of flight and model microgravity studies as shown in Fig. 14.3. This indicates the experimental replicates with the treatment types clustered together and apart from the other experimental treatment type replicates (LSMMG, $1 \times g$, Flight, 25 °C, flight 37 °C).

For two separate analyses comparing the direct analysis of flight versus ground samples and the comparison between spaceflight and LSMMG experiments there were a total of 297 genes (108 up-regulated and 189 down-regulated) that described the overall gene expression alterations occurring during spaceflight (Unpublished
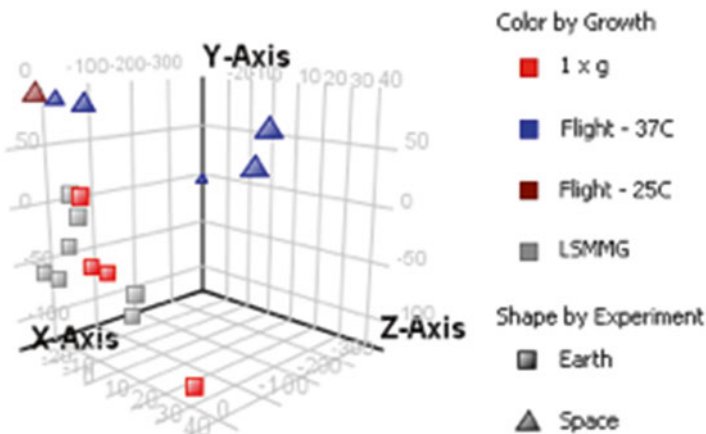
Fig. 14.3 Principal components analysis generated by GeneSpring after Lowess normalization. The colors represent the different experimental replicates (*gray = S. pneumoniae* cultured under LSMMG, compared to RWVs gravity controls; *blue = S. pneumoniae* grown in flight compared to Earth controls), and shapes represent the two different experiments (*squares =* LSMMG experiments conducted entirely on Earth; *triangles =* shuttle/ISS experiments), as indicated in the legend to the right. The *X*-axis, *Y*-axis, and *Z*-axis components represent 50.75 %, 31.5 %, and 10.42 %, respectively, of the total variability between samples

Data). These genes were identified based on their reproducibility across Space Shuttle flights and their specificity to spaceflight as opposed to those seen under modeled microgravity conditions. These genes belonged to diverse functional groups as shown in Fig. 14.4. Electron transport components were down-regulated in space culture conditions compared to ground-based controls. Genes which function in amino acid and nucleotide synthesis and carbohydrate and cell wall synthesis were also altered. Detailed analysis of individual genes is under review for publication elsewhere. In *Salmonella* [9]*, Pseudomonas* [11, 14]*, and Staphylococcus* [5], the *hfq* gene is implicated in the regulation of gene expression in response to
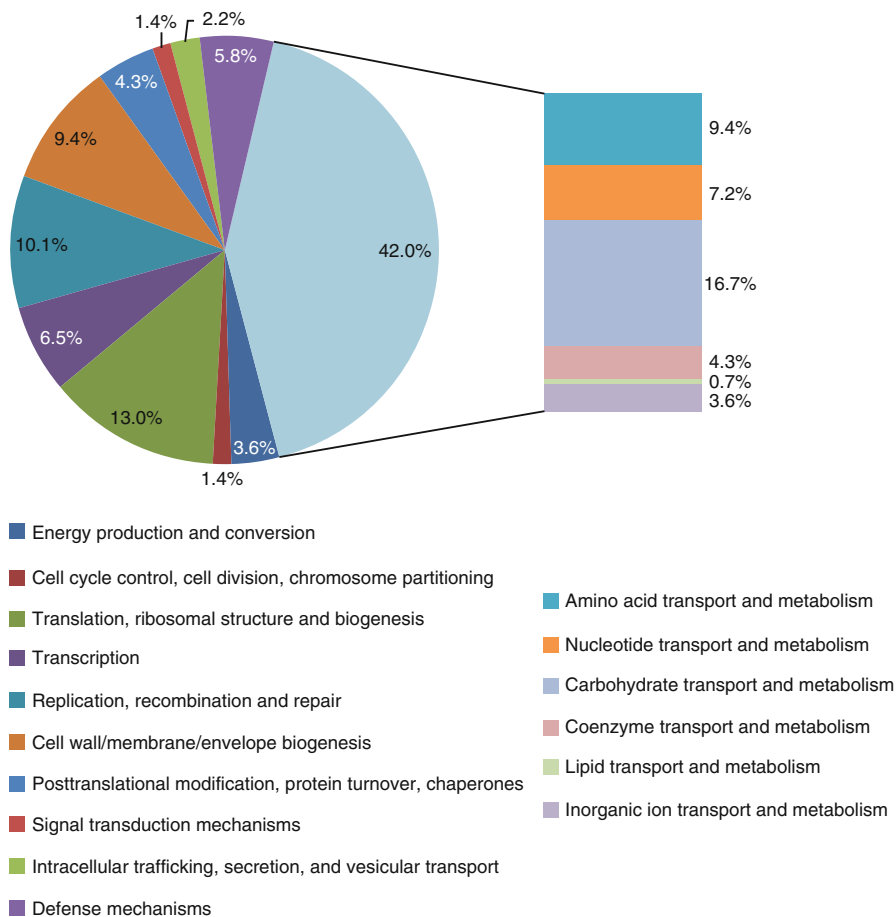
Fig. 14.4 Functions of genes differentially expressed by *S. pneumoniae* grown in space, compared to the bacteria cultured on Earth are shown. Percentages indicate the proportion of altered genes included in the functional category shown, as indicated by color in the legend. Genes with unknown functions were excluded

modeled microgravity and/or true microgravity conditions. Interestingly, *hfq* or an equivalent is not found in the genome of *S. pneumoniae*, and, therefore, the regulation of bacterial gene expression in this microorganism in response to the space environment will be unique from that observed for these other species. The nature of microgravity-induced alterations in the molecular regulation of *S. pneumoniae* will require additional investigation.

14.4   Other Gram Positive Species

Several Gram positive species have been the focus of previous spaceflight and spaceflight analogue studies. Many of these studies provided information into phenotypic changes that could impact disease and its treatment during spaceflight. For example, a small number of spaceflight studies noted an increase in cell concentrations of *Bacillus subtilis* when cultivated in the spaceflight environment as compared to ground controls [3, 45], although this research did not lend itself to identifying a mechanism behind these changes. Of particular relevance, the transfer of mobile genetic elements (MGE) in *Bacillus thuringiensis* has been documented in a study performed aboard the ISS, and it has been hypothesized that MGE exchange may be more efficient in-flight for this microorganism as compared to ground cultures [46]. Confirmation that MGEs can readily be exchanged in the microgravity environment holds value in our understanding of crew health risk as it reveals the potential for endogenous flora of the spacecraft to undergo genetic exchange, presenting an increased risk for antibiotic resistance to occur. Studies using the RWV have focused on bacterial function, such as antibiotic production by *Bacillus brevis*, which was found to be unaffected by LSMMG conditions [47].

Gram positive microorganisms have also been investigated to better understand changes in cellular growth kinetics and culture densities in spaceflight cultures. *B. subtilis* displayed a shortened lag growth phase and an extended exponential growth phase in response to microgravity culture, yielding an increase in final cell density as compared to ground cultures [3]. Kacena et al. also investigated the growth of *B. subtilis* during spaceflight culture and also noted a decreased lag growth phase, that could be temperature dependent, and greater final cell concentrations [45]. Interestingly, Kacena et al. had also investigated the growth of *B. subtilis* in a liquid medium compared to culture of *B. subtilis* on a semi-solid agar [4]. On the semi-solid agar, no differences in growth were observed compared to control cultures suggesting the differences in growth were the result of external physical forces, such as fluid dynamics or extracellular transport, rather than cellular responses directly reacting to gravity [4].

Many spaceflight and spaceflight analogue studies with Gram positive microorganisms have focused on secondary metabolites, especially antibiotic production [48]. In a series of RWV experiments Fang, et al. reported that production of cephamycin C from *Streptomyces clavuligerus* and rapamycin from *Streptomyces hygroscopicus* were inhibited by culture in LSMMG [49, 50]. This inhibition was

similar to repressed production of microcin B17 observed in the Gram negative *Escherichia coli* when cultured in LSMMG [6]. Conversely, production of gramicidin S by *Bacillus brevis* cultured in LSMMG was unaffected compared to control cultures [7]. Interestingly, the sites of accumulation for microcin B17 and rapamycin differed depending on whether the bacteria was cultured in the RWV (extracellular localization) or in standard shaking flasks (intracellular localization), and that this localization could be altered by increasing the fluid shear stress in the RWV by the addition of a single glass bead. Investigation of secondary metabolite production in true spaceflight has also provided interesting findings. Benoit et al. reported initial increased production of actinomycin D by *Streptomyces plicatus* in response to spaceflight culture when compared to ground controls. However, this increase was only observed early in the experiment (days 8 and 12), and spaceflight production was consistently below ground-based control for the remainder of the 72-day experiment [51]. Recent spaceflight experiments investigating *Nocardia mediterranei* aboard Shenzhou III [52] and *Streptomyces avermitilis* aboard Shenzhou VII [48] also showed the potential for altered secondary metabolite production during spaceflight culture.

14.5   Conclusion

Gram positive microorganisms are commonly isolated aboard spacecraft [17, 53] **and many species pose a clear risk to the crew of these vehicles**. As current research provides evidence that these organisms are able to sense and respond to the environment of spaceflight and spaceflight analogues, understanding the stimulus/stimuli that triggers these altered responses and the impact on their phenotype is important in protecting the health of the crew on long duration missions. Evidence from spaceflight experiments has also shown us that Gram positive microorganisms respond in unique ways compared to their terrestrial counterparts. These novel responses provide the opportunity to not only benefit space travelers, but also enable the discovery of previously unknown microbial disease mechanisms that could lead to improved health care to the general public on Earth.

Questions for Future Research
– How do the structural differences between Gram positive and Gram negative bacteria affect their response to microgravity?
– As each microorganism can have markedly different phenotypic responses to spaceflight analogue (and possibly spaceflight) environments, do their biochemical and transcriptional responses also vary?
– As many Gram positive pathogens can be carried by crewmembers during spaceflight missions (either as commensals or subclinical pathogens), what is the crew health risk during long term missions (e.g., to Mars)?

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