Extremophiles in Spacecraft Assembly Cleanrooms

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10.1 Introduction

10.1.1 Planetary Protection

The major goal of planetary protection is to avoid any compromise of scientific exploration of celestial bodies by (biological) contamination. This includes (a) the protection of extraterrestrial ecosystems from terrestrial biomolecules and life forms and (b) the protection of the integrity of missions to avoid false-positive signals from, e.g., life-detection instruments. Besides forward contamination of celestial bodies, a reverse contamination of Earth by extraterrestrial material is also a fundamental concern: "States parties shall pursue studies of outer space, including the Moon and other celestial bodies, and conduct explorations of them so as to avoid their harmful contamination and also adverse changes in the environment of the Earth resulting from the introduction of extraterrestrial matter and, when necessary, adopt appropriate measures for this purpose" (UN Outer Space Treaty 1967; Conley 2011). This scope of the planetary protection policy emphasizes that forward contamination by terrestrial life and even by biomolecules needs to be avoided in order to preserve extraterrestrial bodies and to prevent confounding of future life-detection experiments on other planets (Kminek and Rummel 2015). According to the COSPAR (Committee of Space Research) recommendations, space missions are divided in five categories, considering the scientific interest and also the probability of possible contamination of other planets and, in case of return missions, also Earth (Table 10.1).

Landing missions on Mars are generally assigned to category IV with subcategories a, b, and c. Although Mars appears hostile for life, some so-called special regions could support Earth or own indigenous Mars life, should it exist (Rummel et al. 2014; Kminek and Rummel 2015; Rettberg et al. 2016). The parameters for Martian special regions are currently defined as follows: water activity between 0.5 and 1.0 and 25 °C as the lower limit for temperature (Kminek and Rummel 2015). However, those special regions remain currently untackled by lander missions, due to extremely strict protection regulations.

There is great fear that in particular the search for life could be affected by the contamination of landing spacecraft and their sensitive biosensors. False positives could possibly mask present signatures of Martian life and therefore inhibit the successful search for extraterrestrial life forms. Additionally, although not very likely, Earth organisms could possibly proliferate and therefore contaminate Mars' biotopes, competing with potential indigenous life.

Table 10.1 Planetary protection mission categories (Kminek and Rummel 2015)

Category	Mission type	Possible target
I	Missions to a target body without direct interest for understanding the process of chemical evolution or the origin of life. Since no protection of these bodies is warranted, no planetary protection requirements are necessary	Venus, undifferentiated asteroids
II	Missions to target bodies with significant interest relative to the process of chemical evolution and the origin of life, but in which there is only a remote chance of contamination. Planetary protection requirements include mainly simple documentation and passive contamination control (cleanroom assembly)	Jupiter, Saturn, comets, Uranus
III	Flyby and orbiter missions, targeting a body of significant interest for chemical evolution and/or origin of life, with high risk of contamination that could jeopardize future search for life missions. COSPAR requirements are a documentation that include also a possible bioburden reduction if necessary. Furthermore, an inventory of the microbial community present is required if an impact is very probable	Mars, Europa, Enceladus
IV	Mostly probe and lander missions, targeting bodies of high interest concerning chemical evolutions and/or origin of life, with a significant chance of contamination. Category IV lander missions are separated into three subcategories (a, b, c) with different requirements based on the location of the landing site and the objectives of that mission. IVb and c have the strictest bioburden limits and require detailed documentations, bioassays for bioburden measurements, (partial) sterilization of hardware, aggressive cleaning, protection from recontamination, and aseptic assembly	Mars, Europa, Enceladus
V	All Earth return missions, distinguishing unrestricted and restricted Earth return, depending on the probability of the presence of indigenous life forms on the visited solar body. Restricted Earth return missions require strict containment of samples	Unrestricted Earth return: Moon; restricted Earth return: Mars, Europa

Thus, complete sterility of a spacecraft is a desirable goal. However, nowadays sensitive instruments and detectors onboard do not allow to heat-sterilize the entire spacecraft as done for the Viking lander in the 1970s (111.7 °C ± 1.7 °C, 23–30 h; Puleo et al. 1977). Instead, all assembly procedures are performed in microbiologically controlled cleanrooms, including the integration of pre-cleaned and presterilized spacecraft hardware.

10.1.2 Cleanrooms

In order not to affect or even to confound future life-detection missions on celestial bodies, which are of interest for their chemical and biological evolution, spacecraft

are constructed in so-called cleanrooms and are subject to severe cleaning processes and microbiological controls before launch (Crawford 2005). Cleanrooms are certified according to ISO14644-1. For instance, the cleanroom class ISO 5 corresponds to the former cleanroom class 100 (US FED STD 209E), allowing a maximum of 3.5 particles with a maximum 0.5 μm diameter per liter air.

During assembly, test, and launch operations (ATLO) of, e.g., Mars landers, appropriate cleanliness and sterility levels have to be guaranteed: The proper maintenance of the cleanroom includes a repeated cleaning with antimicrobial agents, particulates are filtered from the air (HEPA filtering), and even staff, working in the cleanroom, must take appropriate actions to minimize the particulate and microbial contamination. Cleanroom personnel have to follow specific access procedures (air locks, tacky mats) to minimize the influx of particulate matter. Staff has to wear special suits, use sterile tools, observe possible biocontamination risks, and even undergo frequent health checks.

Spacecraft assembly cleanrooms are quite similar to pharmaceutical or hospital cleanrooms. In the pharmaceutical industry, for aseptic production, cleanrooms are required, and monitoring microbial and also particle counts are part of good manufacturing practices (Nagarkar et al. 2001).

10.1.3 Contamination Control and Examinations

To date, space missions in preparation have to follow an implementation plan describing all actions necessary to reduce and measure bioburden. This plan also includes the requirement of (daily) sampling of the spacecraft and hardware using swabs and wipes. The recommended sampling size for swabs is 25 cm^2 only, whereas polyester wipes are used for the sampling of larger surfaces. The bacterial spore count is then assessed by culturing a heat-shocked sample according to a standard protocol and aims to reflect the most resistant component of the aerobic, heterotrophic, and mesophilic microbial community present ("bioburden," Administration NASA, Technical Handbook 2010). The current NASA standard is based on the methods that were originally developed for the Viking missions in the 1970s. In brief, the surface of a spacecraft is either swabbed (cotton swabs) or wiped (for larger surfaces). The sampling tools are extracted in liquid, by a combination of vortexing/shaking and sonication. After a heat shock (80 °C, 15 min), the suspension is plated on TSA plates and incubated at 32 °C. After a final count (72 h), the resulting plate count is used as a basis for the calculation of the overall microbial cleanliness of the spacecraft surface.

ESA's new standard methodology is based on the usage of the nylon-flocked swab instead of cotton swabs. Additionally, for improved cultivation of low-nutrient adapted cleanroom microorganisms, the cultivation medium used is R2A instead of TSA, as given in the new ESA standard protocol (2008). If space hardware surfaces are contaminated above the accepted levels, biocleaning is necessary: alcohols (IPA), disinfectants, and UV exposure are some methods applied to reduce the present contaminants. Furthermore, bioshields can be used to enclose certain (clean) hardware or the entire spacecraft to avoid contamination (Debus 2006).

In the case of Mars, limits on bioburden are based on requirements first imposed on the Viking missions. Based on these data, the acceptable microbial contamination for a category IVa mission is limited to 3×10^5 bacterial spores per Mars landing spacecraft or 300 spores per m^2 surface (Viking presterilization biological burden levels; Pillinger et al. 2006). For instance, for Beagle 2, an ESA IVa mission, the overall surface bioburden was estimated to be 2.3×10^4 spores, the total bioburden was 1.01×10^5 spores, and bioburden density was approximately 20.6 spores/m^2 and therefore within the acceptable range (Pillinger et al. 2006).

10.1.4 Cleanroom Microbiology

Examination protocols for spacecraft assembly cleanrooms focus on the detection and enumeration of cultivable, mesophilic, heterotrophic organisms. Nevertheless, cleanrooms are unique environments for microbes: due to low nutrient levels, dry and clean conditions, and constant control of humidity and temperature, these environments are inhospitable to microbial life and even considered "extreme" (Venkateswaran et al. 2001).

Several procedures keep contamination from the outside as low as possible, but these conditions are also highly selective for indigenous extremotolerant microbial communities (Crawford 2005). For space missions, it is crucial to generally control the contaminating bioburden as much as possible. But on the other hand, for the development of novel cleaning/sterilization methods, it is also important to identify and characterize (understand) the microbial community of spacecraft cleanrooms.

The low biomass is generally problematic for microbiological surveys, since the sampling procedure itself is biased and characterized by significant losses during the procedure (Probst et al. 2010a). Furthermore, it is estimated that only 0.1–1 % of all microbes present in one biotope can be cultivated using standard cultivation techniques, increasing the unseen microbial portion in cleanrooms significantly (Amann et al. 1995).

An increasing body of literature about the microbial diversity in spacecraft-associated cleanrooms and on spacecraft hardware is available, including extensive cultivation workup to studies based on next-generation sequencing.

The first article about the microbial analyses of the two Viking spacecraft reported that about 7000 samples were taken from the spacecraft surface during prelaunch activities in order to determine the cultivable microbial load (Puleo et al. 1977). Besides human-associated bacteria (including opportunistic pathogens), which were predominant among the microbes isolated from these samples, aerobic spore-forming microorganisms (*Bacillus*) were found frequently on spacecraft and within the facilities. The predominance of human-associated microorganisms and sporeformers has been confirmed in subsequent publications (e.g., Moissl et al. 2007; Stieglmeier et al. 2009), whereas the portion of *Bacillus* and, e.g., *Micrococcus* was reported to be substantial. In general, 85 % of all isolated microorganisms of the NASA JPL group until 2005 were identified as Gram-positive bacteria

(Newcombe et al. 2005). All of these cultivated microorganisms were obtained from isolation attempts on heterotrophic full media. However, chances of survival of space flights are higher for organisms that can thrive under more restrictive and extreme conditions.

In preparation of missions that intend to land on Mars (such as ESA's ExoMars) or other celestial bodies but also for future lander and Mars Sample Return (MSR) missions, the knowledge about the biological contamination in spacecraft assembly, integration, testing, and launch facilities is an important prerequisite.

In a recent, separate study, two European and one South American spacecraft assembly cleanrooms were analyzed concerning their microbial diversity, using standard procedures, new cultivation approaches, and molecular methods, with the aim to shed light onto the presence of planetary protection-relevant microorganisms within their facilities. This study served as a preparation study for the current ExoMars mission and included the microbial analysis of the Herschel Space Observatory (launched in May 2009) and its housing cleanrooms during ATLO activities at three different locations. Although Herschel did not demand planetary protection requirements, all cleanrooms were under full operation when sampled. The following table gives details about the sampling locations and their characteristics (Table 10.2).

The cultivation procedures and media are summarized in Table 10.3 (see also: Stieglmeier et al. 2009; Moissl-Eichinger et al. 2012).

Table 10.2 Sampling locations and specifics

	Friedrichshafen 1 (FR1)	Friedrichshafen 2 (FR2)	ESTEC (ES)	Kourou (KO)
Location	Friedrichshafen, EADS (European Aeronautic Defense and Space Company)	Friedrichshafen, EADS (European Aeronautic Defense and Space Company)	ESTEC (European Space Research and Technology Centre), Noordwijk	Centre Spatial Guyanais (CSG), Kourou
Sampling date	Apr-07	Nov-07	Mar-08	Apr-09
Cleanroom facility	Hall 6, room 6101–04	Hall 6, room 6101–04	Hydra	BAF
Cleanroom specifics	ISO 5[a]	ISO 5	ISO 8	ISO 8
Sampled surfaces	Various cleanroom surfaces, e.g., floor, stairs, door knobs; spacecraft	Various cleanroom surfaces, e.g., floor, stairs; spacecraft	Various cleanroom surfaces, mainly floor; spacecraft	Various cleanroom surfaces, mainly floor; floor of Ariane5 container

Further details are given in Stieglmeier et al. (2009); Moissl-Eichinger (2011)
[a]Cleanroom was nominally operated at ISO 5 but opened to ISO 8 section just before sampling

Table 10.3 Assortment of media used for the enrichment of cleanroom microbes.

Target microbes	Basic medium	Supplemented with/modification/conditions	Gas phase	"Extreme conditions"
Oligotrophs	R2A	Diluted 1:10, 1:100	Ae	1:100
Alkaliphiles	R2A	pH 9, 11	Ae	pH 11
Acidophiles	R2A	pH 5, 3	Ae	pH 3
Autotrophs	*MM* (methanogenic archaea medium)[a]		H_2/CO_2	Carbon present as CO_2 only
	AHM (autotrophic homoacetogen medium)[a]		H_2/CO_2	
	ASR (autotrophic sulfate- reducer medium)[a]		H_2/CO_2	
	AAM (autotrophic all-rounder medium)[a]		N_2/CO_2	
Nitrogen fix	*N2 fix* (Hino and Wilson N2-free medium)[a]		N_2, N_2/O_2	N_2 as nitrogen source
Anaerobes	TGA (thioglycolate agar)[a]		N_2	Anaerobic medium
	TSA (trypticase soy agar)[a]			
	SRA (sulfate-reducer agar)[a]			
	TS (trypticase soy medium)[a]			
	TG (thioglycolate medium)[a]			
Thermophiles	R2A	Incubated at 50/60 °C	Ae	10 °C
Psychrophiles	R2A	Incubated at 10/4 °C	Ae	4 °C
Halophiles	R2A	Addition of 3.5 % and 10 % (w/v) NaCl	Ae	10 % NaCl
Additional media:				
Heterotrophs	R2A		ma	
Archaea	*MM* (methanogenic Archaea medium)[a]	Sodium acetate, methanol	N_2/CO_2	
	ASM (Archaea supporting medium)[a]	Antibiotics mixture; NH_4Cl or yeast extract	N_2, ma, ae	

Liquid media are given in italics. Abbreviations: *Ae* aerobic, *ma* microaerophilic (<3 % O_2). The last column indicates the extreme conditions applied that are discussed in this article. If not given otherwise, the incubation temperature was 32 °C

[a]Medium recipe and preparation given in Stieglmeier et al. (2009)

It shall be mentioned that most of the isolates obtained from planetary protection-relevant cleanrooms and spacecraft are collected in public culture collections. One, the ESA collection, is maintained by the German Collection of Microorganisms and Cell Cultures DSMZ (Moissl-Eichinger et al. 2012); the other one, for isolates obtained during the NASA Phoenix mission specifically, is maintained at the US Department of Agriculture's Agricultural Research Service Culture Collection in Peoria, Illinois (Venkateswaran et al. 2014).

In the following chapters, results from cultivation attempts performed during the Herschel ATLO activities, focusing on the extremophilic microbial community in spacecraft assembly cleanrooms, will be displayed comprehensively and compared to obtained results from US studies.

10.2 Extremophiles and Extremotolerants: Definition

Generally, extremophiles are microorganisms that require extreme conditions for growth. For instance, psychrophiles are adapted to low-temperature environments and require temperatures lower than 15 °C for optimal proliferation. A cleanroom itself is an extreme environment but hosts mainly extremotolerant microorganisms, accepting the extreme circumstances but preferring moderate conditions for growth. In the following, the author will (for simplifying the terminology) not distinguish between real extremophiles and extremotolerants: For instance, the term alkaliphiles concerning cleanroom isolates includes also microorganisms that tolerate but don't require alkaline conditions.

10.3 Spore-Forming Microorganisms

10.3.1 Background

Spores are resting states of certain bacteria and are usually formed when the organism recognizes a lack in nutrients, such as the carbon or nitrogen source. Endospores of *Bacillus subtilis* are highly resistant to inactivation by environmental stresses, like biocidal agents, toxic chemicals, desiccation, pressure, temperature extremes, higher doses of UV, and ionizing radiation (Nicholson et al. 2000, 2005). Spores possess thick layers of coating proteins, and even their DNA is protected by small proteins (SASPs, Moeller et al. 2008). The gel-like core of a desiccated spore contains only 10–25 % of the water available in a vegetative cell. Enzymes and therefore the metabolism of a spore are more or less inactive. Spores can survive hundreds or maybe even million years, when kept dry and protected against mechanical forces and lethal doses of radiation (Cano and Borucki 1995).

The germination generally needs an activator, e.g., moderate heat. In culture, amino acids like alanine seem to support the germination process. In total, almost

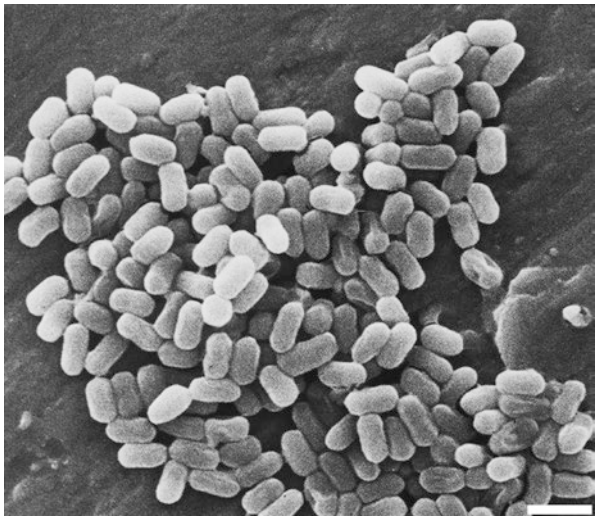

Fig. 10.1 *Bacillus* spores on a spacecraft relevant surface (Probst et al. 2010a), scanning electron micrograph. Bar: 2 µm

20 bacterial genera are able to form spores, but only *Bacillus* and *Clostridium* spores have been subjected to deeper characterization studies (Fig. 10.1).

The multiple resistance properties of such spore-forming microorganisms make them ideal candidates for the survival of a space flight. Additionally, commonly applied sterilization conditions of dry heat or chemical disinfectants that do not harm the spacecraft and its hardware are not able to kill most bacterial spores (Crawford 2005). Since the microbial analysis of the Viking mission has proven the presence of a broad diversity of spore-forming microorganisms on spacecraft surfaces, the main focus of attention has been on them for the past decades.

Although 99.9 % of all *B. subtilis* spores were killed when exposed to a few minutes of Mars simulated surface conditions (in terms of UV irradiation, pressure, gas composition, and temperature), it has also been shown that dried spores were resistant to UV inactivation when mixed with Mars surrogate soil (Schuerger et al. 2003; Crawford et al. 2003; Osman et al. 2008). They were even resistant to sterilizing UV, as long as protected by a shallow layer of sand (Crawford et al. 2003). It therefore can be assumed that highly resistant spores, delivered to Mars, could survive the travel to and the stay on Mars without further damage when located on lander parts not fully exposed to radiation or covered by a thin layer of dust (Osman et al. 2008).

For the selective enrichment of spore-forming microbes, a heat shock at 80 °C for 15 min is one important step within the bioburden-level determination procedure. Besides the effect that most vegetative cells are killed at 80 °C, this heat step is also helpful in stimulation of *Bacillus* spores to germinate. Newcombe et al. (2005) reported that members of the genus *Bacillus* were the predominant microbes among the heat shock survivors; nevertheless the isolation of heat shock-resistant *Staphylococcus*, *Planococcus*, and *Micrococcus* also has been reported (Venkateswaran et al. 2001; Moissl-Eichinger et al. 2013).

For the sake of completeness, it shall also be mentioned that some vegetative microbial cells can resist very harsh conditions such as extreme doses of (UV and ionizing) radiation and desiccation (e.g., *Deinococcus radiodurans*, *Halobacterium* sp. NRC-1; Cox and Battista 2005; DeVeaux et al. 2007). Nevertheless, the information about vegetative, resistant bacteria from spacecraft assembly cleanrooms is very limited.

For sure, the space agency's standard procedures are not able to cover the broadest diversity of tolerant microbes but give a number and a proxy estimation to work with.

10.3.2 Results

Bacillus is a typical spore-forming contaminant in spacecraft assembly cleanrooms. Already Puleo et al. (1977) reported the detection of more than 14 different *Bacillus* strains on the Viking spacecraft.

Newer studies from spacecraft assembly cleanrooms confirm the presence or even predominance of spore-forming bacteria in cultivation assays based on full, heterotrophic media. Six different *Bacillus* strains have been detected on the Mars Odyssey spacecraft (La Duc et al. 2003), some of them revealing resistances against 0.5 Mrad γ-radiation, 5 % H_2O_2 (60 min), or higher doses of UV. In another study, further spore-forming microorganisms, like *Sporosarcina*, *Paenibacillus*, *Actinomycetes*, and *Aureobasidium*, have been detected (La Duc et al. 2004). In newer studies, in particular of the microbial analysis of the Phoenix spacecraft, the presence of (extremophilic) microbial sporeformers has been confirmed (Ghosh et al. 2010), and some new species have been described, such as *Bacillus horneckiae* (Vaishampayan et al. 2010). Another spore-forming bacterium from a genus besides *Bacillus*, *Paenibacillus purispatii*, was isolated from a spacecraft assembly cleanroom at ESA ESTEC (European Space Research and Technology Centre) (Behrendt et al. 2010).

In the here presented study of three cleanrooms, 32 different culture media were used to target a wide range of different microorganisms (see Table 10.3). With this approach, the presence of a broad variety of spore-forming microorganisms in spacecraft assembly cleanrooms was obtained. *Bacillus* and *Paenibacillus* were found in every facility. Overall 13 different *Bacillus* strains, 11 different paenibacilli, *Brevibacillus*, *Clostridium*, *Desulfotomaculum*, *Geobacillus*, *Micromonospora*, *Sporosarcina*, and two *Streptomyces* species were isolated. In general, spore-forming microorganisms accounted for about 25 % of all microbes, but this portion was highly depending on the cultivation strategy (Moissl-Eichinger et al. 2013). The lowest percentage of sporeformers was found during the second Friedrichshafen sampling. During that, the cleanroom was operated at ISO 5, resulting in a higher percentage of human-associated microorganisms and a lower percentage of sporeformers. Most of the spore-forming bacteria observed are associated with environmental biotopes (like soil) and therefore most likely introduced on items moved into the cleanrooms or attached to humans and clothes. It can be concluded that the

higher the operational cleanliness of a facility, the less spore-forming microorganisms could be expected.

B. pumilus SAFR-032, an isolate originally obtained from a class 100 K (ISO 8) cleanroom at the Jet Propulsion Laboratory Spacecraft Assembly Facility (JPL-SAF), was described to form spores with extraordinary UV resistance outcompeting even a standard dosimetry strain of *B. subtilis* (Newcombe et al. 2005). A larger percentage of SAFR-032 spores was found to be even able to survive exposure to dark space conditions (EuTEF, European Technology Exposure Facility) outside of the international space station for 18 months (Vaishampayan et al. 2012).

Different strains of *B. pumilus* have very frequently been isolated from US spacecraft assembly cleanrooms, and many of them were described to possess amazing resistances against H_2O_2 (Kempf et al. 2005) or UV (Newcombe et al. 2005; Link et al. 2004). Generally, these cleanroom isolates revealed a higher resistance to UV irradiation than the type strain of *B. pumilus* (Newcombe et al. 2005).

Strains of *B. pumilus* have been isolated also from the first Friedrichshafen and the Kourou sampling, but these strains were underrepresented (0.6 and 1.4 %, respectively) among all isolates obtained. The most frequent *Bacillus* strains obtained were *B. megaterium* or bacilli affiliated to the *B. thuringiensis/cereus* group. As reported by Newcombe et al. (2005), from 125 aerobic strains isolated from US spacecraft assembly facilities, 65 % were resistant against the heat shock implemented by the NASA standard protocol. Among 15 different *Bacillus* identified, *B. licheniformis* (25 %) and *B. pumilus* (15 %) were the most prevalent species.

10.4 Oligotrophic Microorganisms

10.4.1 Background

Oligotrophs (or oligophilic microorganisms) are microbes that are adapted to low nutrient conditions. Standard laboratory media are usually fully heterotrophic media providing a broad variety of carbon and other nutrient sources. Nevertheless, most of the microbes thriving in natural biotopes have to deal with nutrient restriction and competition with other organisms. Similarly, cleanrooms are characterized by a significant lack of nutrients. Frequent cleaning and air filtering procedures remove particles that could provide nutritive substances, so that the microorganisms present either have to retreat into a resting state (like spores) or have to adapt their metabolism to the extreme circumstances. To date, NASA's standard procedures recommend the usage of TSA medium (Trypticase soy broth) for the cultivation of microbial contaminants in spacecraft assembly facilities. Nevertheless, a pharmaceutical cleanroom study revealed that the portion of cultivables from the cleanroom production unit could be increased by two orders of magnitude when a low nutrient medium was applied instead of a full medium (Nagarkar et al. 2001). Additionally, if looking for possible hitchhikers to Mars, the search for

microbes adapted to low nutrient conditions is even more reasonable: So far, no complex organic molecules have been detected on the Martian surface or in its atmosphere.

10.4.2 Results

Until now, data on oligophilic microorganisms from spacecraft assembly cleanrooms are rather sparse, and the mentioned study from a pharmaceutical cleanroom has not delivered data about the microbial strains detected (Nagarkar et al. 2001).

Until now, no data have been published with respect to oligotrophic microorganisms from spacecraft assembly cleanrooms, and the aforementioned study from a pharmaceutical cleanroom has not delivered data about the microbial strains which were detected (Nagarkar et al. 2001).

In our recent and ongoing studies, R2A medium was used for various cultivation attempts. This medium was originally developed to study microorganisms inhabiting potable water (Reasoner and Geldreich 1985); it is a low nutrient medium that could stimulate the growth of stressed and slow growing microbes. For the detection of oligotrophs in this here discussed study, R2A medium was applied even in a 1:10 and 1:100 dilution, respectively. Interestingly, a broad variety of bacteria was cultured on R2A 1:100, including *Acinetobacter*, *Balneimonas*, *Brevundimonas*, *Citrobacter*, *Kocuria*, *Microbacterium*, *Micrococcus*, *Moraxella*, *Paenibacillus*, *Sanguibacter*, *Staphylococcus*, *Stenotrophomonas*, and *Streptomyces*, whereas *Paenibacillus* and *Streptomyces* are capable of forming spores.

Interestingly, isolates from the first Friedrichshafen sampling that were grown on 1:10 and 1:100 R2A outperformed the number of cultivables on other (nutrient-rich) media (2.8×10^4 and 4.0×10^4 oligotrophic cultivables per m^2 cleanroom surface). Comparably, the samples from the other cleanrooms revealed a high number of oligotrophs present. Since our approach was the first in this field searching for oligotrophs, further studies in other cleanrooms will be necessary and are highly recommended.

Based on own observations and results from other studies, the new ESA standard for biocontamination measurement relies on R2A instead of TSA for the cultivation of spacecraft assembly-related microorganisms.

10.5 Alkaliphiles and Acidophiles

10.5.1 Background

Although the pH of Mars' regolith was estimated to be rather neutral (7.2± 0.1, Plumb et al. 1993), the presence of alkaliphiles and acidophiles in spacecraft assembly cleanrooms can deliver valuable information for planetary protection considerations and in particular for cleanroom maintenance: Most of the disinfectants and detergents used for (bio)cleaning in such cleanrooms are either pH neutral or

alkaline. It is unclear if the consequent treatment with, e.g., alkaline detergents could result in a positive selection effect. A preference of alkaline or acidic media by the microbial diversity in cleanrooms was analyzed in two independent studies.

10.5.2 Results

Samples from diverse spacecraft assembly cleanrooms were plated on R2A with pH 9, 10.6, and 3 (La Duc et al. 2007), pH 11 and 3 (Ghosh et al. 2010), or pH 9, 11, 5, and 3 (this project; Moissl-Eichinger et al. 2013). A broad variety of bacteria tolerating pH 10.6 were reported to be present in US cleanrooms (e.g., *Bacillus*, *Staphylococcus*, *Sphingomonas*, and many others; La Duc et al. 2007), and also our studies observed various alkaliphiles (pH 9, pH 11; Table 10.4). Interestingly, *Bacillus* and *Brevundimonas* were detected in all studies, accepting a medium pH of 10.6 and 11, respectively. Alkaliphiles were observed in every facility looked at, with numbers ranging from 1.6×10^2 (ISO 6, JSC-GCL) to 2.0×10^6 per m^2 (ISO 8, LMA-MTF). During the Herschel campaign, 4.6×10^2 (second sampling Friedrichshafen, ISO 5) up to 8.4×10^3 (ESTEC, ISO 8) were measured, whereas the number of alkaliphiles in Kourou samples could not be determined due to overgrowth of the agar plates. Similar levels were obtained from the Phoenix assembly cleanroom (up to 68×10^2 CFU per m^2 (Ghosh et al. 2010).

Nevertheless, the detection of acidophiles was much more difficult. The colony counts on agar plates with pH 5 were very low, no isolate was obtained during the Herschel campaign tolerating pH 3, whereas an acid-tolerant colony count was reported for US LMA-MTF and KSC-PHSF facility only (La Duc et al. 2007). All other samplings were negative for acidophiles. Nevertheless, none of the isolates obtained during the US study was analyzed further or revealed multiresistant properties (La Duc et al. 2007).

A substantial preference of alkaline media was found in all facilities analyzed so far. One isolate, *B. canaveralius*, was obtained recently and was found to be salt and alkali tolerant (Newcombe et al. 2009). To date, the reasons for the shifts toward alkaliphily are unclear, but a positive selection by the usage of (alkaline) cleaning detergents seems probable and could result in an outperformance of acidophiles or non-alkali tolerants. If so, the selectivity via the pH of cleaning agents could be circumvented by using detergents with alternating pH.

10.6 Autotrophic and Nitrogen-Fixing Microorganisms

10.6.1 Background

The capability to fix nitrogen from the gaseous atmosphere or to grow autotrophically on CO_2 is an important property of primary producers. The activities of these microbes are the prerequisites for other microorganisms to colonize new nutrient-poor environments (Thomas et al. 2006). Since cleanrooms and also the Martian

environment are depleted in organic materials, primary producers could pave the way for secondary settlers.

Nevertheless, most studies have not reported the attempt to grow primary producers. The majority of studies looking for cultivables from spacecraft and associated cleanrooms have used heterotrophic, solid full media. The assortment of special media used in our studies also included media selectively for chemolithoautotrophs with CO_2 as the only carbon source. Media providing nitrogen only in the gas phase were also applied.

10.6.2 Results

Seven isolated bacterial genera were able to fix CO_2; eight were able to fix N_2. Further details about the isolated bacterial genera are given in Table 10.4.

An interesting observation was that solely species of the bacterial genus *Paenibacillus*, *Micrococcus*, and *Sanguibacter* were able to perform both reactions, whereas *Sanguibacter marinus* was the only species that was isolated on an autotrophic and N_2 fixer medium in parallel (Kourou sampling). The type strain of this species was originally isolated from coastal sediment (Fujian province of China), and none of the observed properties has been reported in the original strain description (Huang et al. 2005). It can be imagined that the cleanroom isolate shows distinct properties due to the adaptation to the extreme biotope or the type strain has not been tested concerning these metabolic capabilities. *Paenibacillus* and *Streptomyces* were the only spore-forming microorganisms that were able to fix N_2 (both) or CO_2 (*Paenibacillus*).

10.7 Anaerobes

10.7.1 Background

The atmospheres of most planets within the reach of space missions contain only traces of oxygen, most likely not enough to support aerobic life as we know it from terrestrial biotopes (Thomas et al. 2006). Since the Martian surface is exposed to radiation and the soil is very oxidizing, the Martian subsurface could be an anaerobic biotope for possible life (Boston et al. 1992; Schulze-Makuch and Grinspoon 2005). On Earth, (facultative) anaerobes are widespread in different environments and can be detected in, e.g., oxic soil, aerobic desert soils, or other biotopes such as the human body (Küsel et al. 1999; Peters and Conrad 1995; Tally et al. 1975). The latter makes them potential contaminants of spacecraft assembly facilities through staff, having close contact to flight hardware.

Generally, there are different types of anaerobic organisms. Facultative anaerobes always prefer aerobic conditions but are able to grow under conditions with or without oxygen; aerotolerant anaerobes do not require oxygen for their growth and show no preference. Strict anaerobes (e.g., methanogens) never require oxygen for

Table 10.4 Extremophilic isolates from global spacecraft assembly cleanrooms

	Oligotrohs [1]	Psychrophiles [2]	Alkaliphiles [3]	Anaerobes	Thermophiles [4]	Halophiles [5]	CO2 fix	N2 fix	Species (location/extremophily)
Acinetobacter	○	○○	○						*A. sp.*, *A. ursingii* (FR2), *A. johnsonii* (KSC/alk,psy)
Actinotalea			○						*A. fermentans* (KO)
Aerococcus				○		○			*A. urinaeequi* (KO)
Agrococcus			○						*A. jenensis* (KSC)
Arsenicicoccus				○			◆		*A. bolidensis* (FR2)
Arthrobacter		○	○	○				◆	*A. sp.* (KSC/alk, KO/an, N_2), *A. polychromogenes* (KSC/psy)
Bacillus		○	○○ ○○ ○○ ○○ ○○	○○ ○○	○○ ○	○			*B. thermoamylovorans* (ES/alk, FR1/an), *B. gibsonii* (FR2/alk), *B. licheniformis* (FR1/alk, ES/an,therm), *B. pumilus* (KO/an, JSC/alk, KSC/alk), *B.* tc (FR2, ES,KO/an), *B. badius* (KO/therm), *B. coagulans* (ES,KSC/therm), *B. megaterium* (KO/halo), *B. sp.* (JSC/alk; KSC/alk,psy), *B. oshimensis* (KSC/alk), *B. pseudoalcaliphilus* (KSC/alk), *B. thuringiensis* (KSC/alk)
Balneimonas	○		○						*B. sp.* (KO)
Blastococcus		○							*B. sp.* (KSC)
Brachybacterium			○						*B. paraconglomeratum* (LMA)
Brevibacillus		○○	○		○				*B. agri* (FR1), *B. invocatus* (KSC/psy)
Brevibacterium			○						*B. frigoritolerans* (ES)
Brevundimonas	○		○○						*B. nasdae* (FR2, KO), *B. diminuta* (KSC/alk)
Cellulomonas				○○			◆		*C. hominis* (FR1, KO/an, KO/aut)
Cellulosimicrobium			○						*C. funkei* (KSC)
Citrobacter	○								*C. werkmanii* (KO)
Clostridium				○					*C. perfringens* (ES)
Corynebacterium				○					*C. pseudogenitalium* (ES)
Cupriavidus				○					*C. gilardii* (KO)
Dermabacter				○					*D. hominis* (FR2)
Desemzia						○			*D. incerta* (KO)
Desulfotomaculum				○					*D. guttoideum* (KO)
Dietzia		○	○						*D. maris* (KSC)
Enterococcus				○○			◆		*E. casseliflavus* (KO/aut), *E. faecalis* (ES/an), *E. faecium* (ES/an)
Facklamia			○	○					*F. sp.* (KO)
Geobacillus					○○ ○○				*G. caldoxylosilyticus* (ES, KSC), *G. stearothermophilus* (JPL), *G. kaustophilus* (JPL), *G. thermodenitrificans* (JSC)
Georgenia		○		○					*G. muralis* (KO), *G. sp.* (KSC)
Herbaspirillum		○							*H. sp.* (KSC)
Janibacter			○						*J. terrae* (KSC)
Kocuria	○		○						*K. rhizophila* (ES), *K. rosea* (KSC)
Labedella		○							*L. kawkjii* (KSC)
Lysobacter			○						*L. sp.* (KO)
Massilia		○							*M. brevitalaea* (KO)
Microbacterium	○		○○ ○					◆	*M. oleivorans* (KO/oligo), *M. paraoxydans* (KO/oligo, N_2), *M. schleiferi* (LMA), *M. aurum* (JSC), *M. arborescens* (KSC), *M. testaceum* (KSC)
Micrococcus	○		○○ ○○			○	◆		*M.* sp. (KO/oligo, alk), *M. flavus* (KO/alk), *M. indicus* (ES/aut) *M. luteus* (KO,FR2,FR1/alk; KO/halo), *M. mucilaginosus* (KSC/alk)
Moraxella	○		○	○					*M. osloensis* (FR2/oligo; FR1/alk,an)
Nocardioides			○						*N. oleivorans* (KSC)
Oceanobacillus			○○ ○						*O.* sp. (JPL/alk), *O. iheyensis* (KSC/alk), *O. profundus* (KSC/alk)
Paenibacillus	○		○○ ○	○○ ○	○		◆ ◆	◆	*P. pasadenensis* (FR1/oligo,alk, N_2; ES/alk), *P. telluris* (ES/alk), *P.* sp. (KO/alk), *P. amylolyticus* (FR1/alk), *P. glucanolyticus* (FR1/alk), *P.* sp. (ES/an), *P. ginsengisoli* (ES/an,auto), *P. barengoltzii* (FR1/an), *P. cookii* (FR1/therm), *P. wynii* (LMA/an)
Paracoccus								◆	*P. yeeii* (ES)
Planctibacter		○	○						*P. flavus* (KSC)
Propionibacterium				○○ ○					*P. acnes* (FR1,FR2, ES), *P. avidum* (ES)
Pseudomonas		○○	○○					◆	*P. luteola* (FR2/ N_2), *P. xanthomarina* (KO/alk,psy), *P. stutzeri* (KSC/alk), *P. oryzihabitans* (KSC/psy)
Rhodococcus		○	○						*R. fascians* (KSC/psy), *R. globerulus* (KSC/alk), *R. kroppenstedtii* (KSC/alk), *R. sp* (KSC/psy)
Roseomonas		○	○						*R. aquatica* (KO)
Sanguibacter	○	○		○			◆	◆	*S. marinus* (KO), *S. sp.* (KSC)
Sphingomonas		○	○○						*S. oligophenolica* (JSC), *S. trueperi* (JSC), *S. dokdonensis* (KSC), *S. yunnanensis* (KSC), *S. sp* (KSC/psy)
Spirosoma		○							*S. aquatica* (KSC)
Staphylococcus	○ ○		○○ ○○	○○ ○○		○○ ○○	◆ ◆		*S. haemolyticus* (ES, FR1/oligo, FR1, FR2, ES/alk, ES,KO/an, FR1, FR2, ES/halo,auto), *S. warneri* (KO/an), *S. pasteuri* (FR2/an, KO,ES/halo), *S. lugdunensis* (FR2/an), *S. hominis* (FR1/halo), *S. epidermidis* (JSC/an)

Stenotrophomonas	○		○○	○				◆	*S. maltophilia* (FR2), *S. rhizophila* (KSC/alk)
Streptomyces	○		○					◆	*S. luteogriseus* (ES)
Tessaracoccus				○					*T. flavescens* (KO)
Variovorax		○							*V. paradoxux* (KSC)

Circles stand for each location, where detected. Black: isolates from this study. Abbreviations of locations according to Table 10.2. Red: for comparison, isolates from US studies are listed, too (La Duc et al. 2007; Ghosh et al. 2010); locations: KSC (Kennedy Space Center), JSC (Johnson Space Center), LMA (Lockheed Martin Aeronautics), JPL (Jet Propulsion Laboratory). Further abbreviations: an (anaerobic), alk (alkaliphilic), oligo (oligotrophic), therm (thermophilic), halo (halophilic), aut (autotrophic), N2 (N2 fixing), psy (psychrophilic), B. tc (*Bacillus thuringiensis/cereus* group)
[a]Oligotrophs were grown on R2A 1:100.
[b]Psychrophiles were grown on R2A at 4 °C
[c]Alkaliphiles were grown on R2A with pH 10.6 and 11, respectively
[d]Thermophiles were grown at 50, 60 and 65 °C
[e]Halophiles were grown on R2A containing 10 % NaCl

their reproduction and metabolism and can even be growth inhibited or killed by oxygen.

Until now, not much is known about the presence of anaerobically growing microorganisms in spacecraft cleanrooms. The presence of anaerobic microorganisms (enriched using the BD GasPaK system) in surface samples from US cleanrooms has rarely been reported (Ghosh et al. 2010; La Duc et al. 2007). Overall 25 facultatively anaerobic strains were retrieved from the Phoenix housing facility, whereas members of the facultatively anaerobic genera *Paenibacillus* and *Staphylococcus* have been isolated in the course of a study about extremotolerant microorganisms (La Duc et al. 2007).

A proper anaerobic cultivation necessitates the application of the Hungate technique (Hungate 1969). Although this method has undergone a few simplifications during past decades, it still requires specialized equipment and practical experience. During our research, samples from the Herschel campaign were – for the first time – subjected to growth experiments performed with the Hungate technology, and a broad variety of microbes capable of anaerobic growth were isolated (Stieglmeier et al. 2009).

10.7.2 Results

A variety of anaerobic microorganisms was successfully isolated from all four cleanroom samplings. Overall, 30 strains were isolated on anaerobic media. The greatest number and diversity of bacteria were obtained from the Kourou sampling (13 species). The following chart shows the oxygen requirements of isolates obtained from our campaign (see also Table 10.4 and Fig. 10.2).

In most cases, anaerobically enriched species were identified to be facultative anaerobes, comprising 16–78 % of the total counts (numbers were calculated based on own observations and literature review for microbes grown on aerobic plates only; for comparison see Stieglmeier et al. 2009). Colony counts obtained on anaerobic full media showed the presence of up to 5.8×10^2 anaerobes per m^2 cleanroom surface (Kourou sampling).

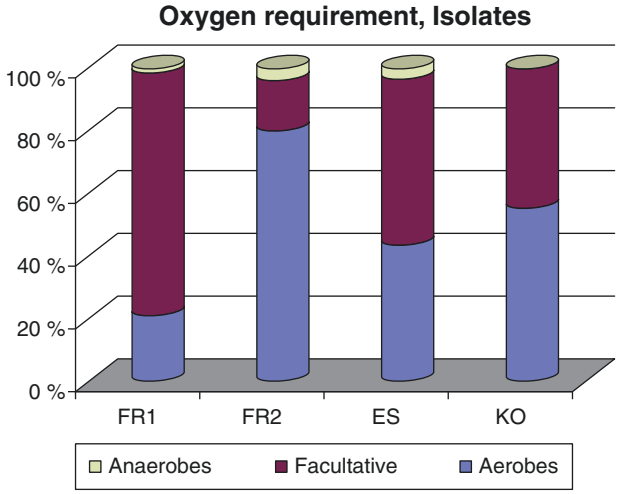

Fig. 10.2 Oxygen requirements of isolates. Physiological capabilities are either based on own experiments or published data (for isolates grown on aerobic media only)

Only a comparatively low percentage of microbes grew strictly anaerobic (*Propionibacterium*, *Corynebacterium*, *Desulfotomaculum*, *Clostridium*; 0.7–4 % of all isolates at each location). *Propionibacterium acnes*, typically found on human skin, was isolated from each European cleanroom and therefore the most prominent strict anaerobe detected. All strict anaerobes except *Desulfotomaculum* were opportunistic pathogens and isolated from full, heterotrophic media. Interestingly, the *Corynebacterium* isolate (*C. pseudogenitalium*) could not be grown under aerobic conditions, although its type strain was described to be facultatively anaerobic (Stieglmeier et al. 2009).

Desulfotomaculum guttoideum was the only strict anaerobe isolated from the Kourou sampling. It was grown on sulfate-reducer specific medium, but without producing black colonies that would indicate a sulfate-reducing activity. As it was clarified in a previous publication, *Desulfotomaculum guttoideum* was misclassified and is actually affiliated to *Clostridium* cluster XIVa (Stackebrandt et al. 1997). This strain is therefore closely related to *Clostridium sphenoides*, a fermentative, saccharolytic, sulfite and thiosulfate (but not sulfate) reducing sporeformer. Other spore-forming microorganisms capable of anaerobic growth were *Bacillus*, *Paenibacillus*, and *Clostridium (Desulfotomaculum)*. Further details are given in Table 10.4.

These data were confirmed by an analysis of the anaerobic microbial diversity in NASA's cleanrooms at the Jet Propulsion Laboratory. This study was based on a microbial enrichment of cleanroom samples under anaerobic conditions, which was then analyzed via cultivation, 16S rRNA gene sequence analysis, and microarray (Probst et al. 2010b). *Clostridium* and *Propionibacterium* were the only strictly anaerobic microbes isolated, whereas additionally *Oerskovia*, *Dermabacter*, *Bacillus*, *Granulicatella*, *Sarcina*, *Leuconostoc*, *Paenibacillus*, *Staphylococcus*, and *Streptococcus* were detected during the molecular approach (Probst et al. 2010b).

Our results indicate that the facultatively and strictly anaerobic microbial community is quite diverse and maybe even dominant in spacecraft assembly cleanrooms.

10.8 Thermophiles and Psychrophiles

10.8.1 Background

The Martian surface is very cold. Although the temperatures can reach up to 20 °C in certain areas in the summer, the average temperatures lie around and way below 0 °C. Actually, also Earth's biosphere is quite cold – more than 70 % of its water occurs as ice, and the world's oceans reveal temperatures below 5 °C (National Research Council 2006). A typical terrestrial biotope used for comparative studies is the permafrost environment exhibiting a lively, highly diverse microbial community. It is assumed that Earth's psychrophiles could survive under certain circumstances in the Martian environment but would grow very slowly (National Research Council 2006).

Although (hyper)thermophiles would probably not be able to proliferate on Mars, this group of microorganisms is often employed in studies concerning the origin and the evolution of life. Hot conditions prevailed on early Earth, and many thermophiles have "basic" metabolic capabilities (for instance, chemolithoautotrophy). Thermophiles are also generally considered more resistant toward environmental stresses than moderate or cold-loving microorganisms.

For these reasons, experiments were carried out searching for microorganisms in cleanroom environments that could grow under significantly higher or lower temperatures than the standard incubation temperature for all other experiments (32 °C).

10.8.2 Results

The study of US spacecraft assembly cleanrooms reported no growth of microorganisms on R2A medium when incubated at 4 °C for 10 days (La Duc et al. 2007). Differently, psychrophiles were reported to be present in the Phoenix assembly area, where 30 strains were identified that were capable of growth at 4 °C (Ghosh et al. 2010). These strains included *Brevibacillus*, *Bacillus*, *Acinetobacter*, *Pseudomonas*, *Sphingomonas*, *Variovorax*, *Herbaspirillum*, *Arthrobacter*, *Curtobacterium*, *Dietzia*, *Labedella*, *Plantibacter*, *Rhodococcus*, *Blastococcus*, *Sanguibacter*, *Rhodococcus*, and *Spirosoma* species.

Our study was selective for microorganisms capable of growing at 10 and 4 °C. The incubation duration was prolonged up to 3 months. *Acinetobacter*, *Massilia*, *Pseudomonas*, and *Roseomonas* were isolated at 4 °C; additionally, *Bacillus*, *Brevundimonas*, *Micrococcus*, *Moraxella*, *Paenibacillus*, *Sanguibacter*, *Sphingomonas*, *Sporosarcina*, *Staphylococcus*, and *Stenotrophomonas* were observed at 10 °C. *Sporosarcina globispora*, a spore-forming bacterium, even was the only isolate that was not able to grow at 32 °C, the standard cultivation temperature.

Nevertheless, most of the "psychrophilic" isolates also had been obtained by using other cultivation methods at higher temperatures (32 °C), too. It can be assumed that many (most?) of the present microorganisms in cleanrooms are capable of growing at lower (very low) temperatures but that cell proliferation takes significantly longer than under higher, optimal temperature conditions.

The selective enrichment of thermophiles on R2A at 65 °C (La Duc et al. 2007; Ghosh et al. 2010) or 60 and 50 °C (Herschel study) allowed the isolation of three *Geobacillus* and one *Bacillus* strain (La Duc et al. 2007) and *Bacillus*, *Brevibacillus*, *Paenibacillus*, and *Geobacillus*, respectively. Also Ghosh et al. (2010) reported the presence of thermophilic microorganisms before spacecraft had been transferred into the cleanroom.

Interestingly, *Bacillus coagulans* and *Geobacillus caldoxylosilyticus* were found in ESA- and NASA-related cleanrooms, whereas the latter was described as an obligate thermophile ("Saccharococcus caldoxylosilyticus," Ahmad et al. 2000), which is congruent to the observations made in this study (no growth was observed at 32 °C). It is unclear how *Geobacillus* (spores) entered the cleanroom (although not capable to proliferate under the thermal conditions of a cleanroom) and why this organism was detected in two independent studies in cleanrooms on different continents. In general, *Geobacillus* spores have been described to be very resistant to environmental (thermal) stress (Head et al. 2008). Because of this high resistance, it possibly survived strict cleanliness control conditions, after being carried into the facilities by human and item traffic.

10.9 Halophiles

10.9.1 Background

Halophiles have been discussed as possible survivors on Mars, since the Martian liquid water is suspected to contain high concentrations of different salts (Landis 2001). Additionally, a high resistance of salt-crystal-associated halophiles against UV radiation has been reported, making them potential survivors on the Martian surface (Fendrihan et al. 2009). Prevention of potential contamination of the Martian surface with halophiles is therefore highly important. Nevertheless, hardly any studies have been carried out thus far to investigate the potential presence of halotolerants and halophiles in spacecraft assembly cleanrooms. In order to obtain insights into the distribution of these organisms in cleanrooms, samples from two studies were plated on R2A containing different NaCl concentrations.

10.9.2 Results

Samples from US cleanrooms were plated onto R2A containing 25 % (w/v) NaCl, but no growth was observed (La Duc et al. 2007; Ghosh et al. 2010). In contrast, samples obtained from the Herschel study were plated on R2A containing 3.5 and

10 % (w/v) NaCl. R2A containing 3.5 % NaCl revealed that most of the organisms isolated via other cultivation attempts were also capable of tolerating this comparatively low concentration of NaCl. The plates containing 10 % NaCl revealed much lower cell counts: *Aerococcus*, *Bacillus*, *Desemzia*, *Micrococcus*, and *Staphylococcus* were observed on this medium (Table 10.4). The most prevalent species accepting higher concentrations of NaCl were staphylococci mainly originating from human skin, where they are exposed to higher levels of salt. Some staphylococci from this campaign were transferable to salt concentration up to 16 % (w/v). Despite obvious presence of halophilic/halotolerant microbes, *Bacillus megaterium* was the only spore-forming isolate that was detected on salty agar plates.

10.10 Other Extremotolerant Bacteria and Eukarya

To complete the data presented here, other resistances of spacecraft assembly microbes shall be mentioned, although their presence has not been tested for the Herschel campaign. The microbial resistance against radiation (UV and gamma) as well as hydrogen peroxide has been in the focus of interest, since these are techniques usually applied for the sterilization of spacecraft components (besides dry heat).

La Duc et al. (2007) and Ghosh et al. (2010) reported the presence of UV-C (1,000 J m^{-2})-resistant and 5 % hydrogen peroxide-resistant microorganisms in the cleanrooms. These resistances were found for spore-forming microbes mainly (*Bacillus*, *Nocardioides*, *Paenibacillus*), whereas *B. pumilus* was resistant against both conditions.

The detection of H_2O_2-resistant *B. pumilus* has been reported even earlier (Kempf et al. 2005) and in particular multiresistant *B. pumilus* SAFR-032 was studied extensively at the NASA Jet Propulsion Laboratory. The whole genome has meanwhile been sequenced and annotated (Gioia et al. 2007). Although the sequence revealed differences and additional, unknown genes to closely related *B. subtilis* or *B. licheniformis*, the *B. pumilus* genome seems to lack genes functioning in UV or H_2O_2 resistance found in other *Bacillus* strains. Further studies will certainly be necessary to understand the molecular basis of extremotolerance in bacteria.

Interestingly, Eukarya have been detected only sparsely during the Herschel campaign. Solely one representative of *Coprinopsis* (fungi) and a few yeast strains have been isolated on R2A. Nevertheless, the entire study focused on microorganisms, and the conditions certainly would have to be adapted for the cultivation of Eukarya. Although fungi can produce spores, their resistance properties, possible extremotolerance, and resulting impact on planetary protection considerations have not been studied yet and are highly recommended. Nevertheless, the detection of *Aureobasidium pullulans* from spacecraft assembly facilities was reported, surviving 1 Mrad gamma radiation for 5.5 h (Bruckner et al. 2008).

For the sake of completeness, and also the discovery of *Tersicoccus phoenicis*, a member of the bacterial *Micrococcaceae* should be mentioned. Although it was not found to be extraordinarily resistant to one specific stress, this microbe, which

represents a novel genus, was found solely in spacecraft assembly cleanrooms so far, namely, at the Kennedy Space Center (Phoenix cleanroom) and at the Centre Spatial Guyanais in Kourou, French Guiana, during the Herschel campaign (Vaishampayan et al. 2013a). Due to the unusual distribution and the general resistance against overall cleanroom conditions, this microbe was declared as one of the "Top 10 New Species of 2014" by the International Institute for Species Exploration.

10.11 Archaea

10.11.1 Background

Archaea, the third domain of life, have meanwhile been detected in almost any "normal" biotope, like marine and freshwater or soil. For more than 20 years, they were considered extremophiles that were ecologically restricted and highly adapted to specific and often hostile biotopes. Many of the extremophilic archaea have very interesting properties. In the eyes of many researchers, they are "primitive" in their metabolism (which actually means they can act as primary producers), which could be an advantage when settling in new biotopes. Detailed resistance experiments with vegetative (hyper) thermophilic archaea have unexpectedly revealed tolerances against desiccation, vacuum, and UV or gamma radiation (Beblo et al. 2009, 2011). It is unclear, however, whether these organisms could withstand the extremely harsh conditions during space travel, lack of nutrients, or low temperatures in extraterrestrial environments.

The major procedures to detect microorganisms in cleanrooms are still targeting Bacteria, but Archaea are more and more considered a possible microbial contaminant on spacecraft. Although the possibility that Archaea, such as methanogens or halophiles, might be able to survive a space flight and survive or even to thrive on Mars (Taubner et al. 2015; Kendrick and Kral 2006; Landis 2001), the existence of Archaea in human-controlled and rigorously cleaned environments has not been assessed until a few years ago, so that it was unclear if Archaea even could be found in spacecraft-associated cleanrooms.

The vast majority of mesophilic and psychrophilic Archaea still resist cultivation, and also the attempt to cultivate Archaea from spacecraft assembly facilities failed (Moissl et al. 2008; Moissl-Eichinger 2011). For this reason, the detection of Archaea can be solely based on molecular studies, which are presented in the following chapter.

10.11.2 Results

In 2008, we reported the detection of archaeal 16S rRNA gene signatures in two US spacecraft assembly cleanrooms (Moissl et al. 2008; Tally et al. 1975). Using a very sensitive PCR approach, 30 different cren- and euryarchaeal sequences were derived from NASA facilities. Meanwhile, the relevant branch of the Crenarchaeota has been reclassified as Thaumarchaeota (Brochier-Armanet et al. 2008).

The omnipresence of Archaea in global cleanrooms was confirmed in a follow-up study (Moissl-Eichinger 2011) in which Archaea were detected in all cleanrooms analyzed (second sampling Friedrichshafen, ESTEC and Kourou). As already reported in 2008, most of the gene sequences obtained clustered within the Thaumarchaeota group 1b. The closest cultivated neighbor *Candidatus* Nitrososphaera gargensis showed more than 4 % difference in the 16S rRNA gene sequence; most of the relatives from various natural biotopes are still uncultivated. Nevertheless, many representatives of this group were described to possess genes for ammonia oxidation (Pester et al. 2011).

The detection of these Thaumarchaeota in different spacecraft assembly cleanrooms was substantial and led to the hypothesis that these archaeal signatures could be linked to human presence (Moissl-Eichinger 2011), similar to the majority of the cleanroom bacteria that turned out to be human-associated or even opportunistic pathogens (e.g., *Staphylococcus;* Moissl et al. 2007; Stieglmeier et al. 2009). Meanwhile, this assumption could be proven and these Thaumarchaeota were clearly linked to the human microbiome, and the source of these archaeal signatures, the human skin, could be identified (Probst et al. 2013). This finding again confirmed the staff being the major contamination source for microbes in cleanrooms.

Quantitative PCR revealed that the average number of Archaea per m^2 cleanroom surface is around two to three logs lower than the estimated total number of Bacteria. Although the number of Archaea appears quite low, their presence is as consistent as typical bacterial cleanroom contaminants – and they were found to be potentially alive at the time of sampling (Mahnert et al. 2015). In the latter study, the microbiome of a US cleanroom was deeply sequenced, revealing the presence of intact *Cand*. Nitrososphaera cells (Thaumarchaeota) and *Haloferax* (Euryarchaeota). These assumptions were congruent to earlier studies, detecting methanogens and other halophiles additionally (Moissl et al. 2008; Moissl-Eichinger 2011). Due to the potential of some archaea to survive in particular under harsh conditions, and their detection only inside, not outside the cleanroom, they were suggested as indicators of cleanroom environments (Mahnert et al. 2015).

Meanwhile, it is well accepted that also Archaea are part of the cleanroom microbiome, and due to the finding that they are intact and thus potentially alive, a potential threat with regard to planetary protection aspects has to be discussed. However, further studies will be necessary, and additional attempts for cultivation are recommended.

10.12 Lessons Learned from Cleanroom Microbiomes: Extremophiles Are Everywhere

A broad variety of extremotolerant bacteria was successfully isolated from each facility analyzed so far. Microorganisms that were able to grow under extremely oligotrophic, cold, alkalic, anaerobic, warm, and high-salt conditions were detected (Table 10.4). Besides that, we have shown that many microbes thriving or surviving

in cleanrooms are able to fix nitrogen and/or carbon dioxide and could therefore serve as primary producers. The following list summarized the main findings of recent studies:

- Sporeformers were present in each facility analyzed, but the total number did not exceed 25 % of all isolates. The lowest percentage was seen in FR2, the "cleanest" environment sampled.
- The highest cell counts were obtained on media with lower nutrient levels and higher pH, hinting toward an influence of the environmental selective forces.
- Primary producers were found in an unexpected diversity: autotrophs and N_2-fixing microbes were successfully isolated.
- Strictly anaerobic bacteria were successfully isolated but are present in a low number only (up to 4 %), although facultative microbes were found to add up to 78 %.
- Thermophiles, psychrophiles, and halophiles were found in the cleanrooms.
- Archaea were detected in each facility and their presence is significant. The properties of the (thaum-)archaeal cleanroom community are unclear, but Archaea are able to persist and intact cells seem to be present.
- Cleanliness level of a cleanroom definitely influences the microbial diversity. The broadest diversity of cultivables was seen in Kourou samples and ESTEC (both cleanrooms were operated at ISO 8). It is assumed that also the environmental conditions have influence on the microbial diversity within the cleanrooms, since Kourou is located in a very humid environment.
- Most of the microbes detected in the overall study were human associated, but the most resistant strains seem to be environmental organisms.

Furthermore, we have shown that besides *Bacillus*, also other (spore-forming) bacteria can play a significant role in cleanroom environments. For instance, several *Paenibacillus* strains have been detected; at least three of them were identified to be novel isolates. Many bacilli are dependent on complex organic compounds for their metabolism. Interestingly, although closely related, some paenibacilli seem to have the ability of nitrogen and carbon dioxide fixation. First resistance experiments with our isolates revealed a proper capability to survive desiccation, vacuum, heating, or Mars-cycle simulations (Fig. 10.3).

One novel isolate (ES_MS17, candidatus *Paenibacillus purispatii* sp. nov.) was shown to have profound metabolic capabilities in nitrogen conversion processes (Behrendt et al. 2010).

Although paenibacilli have not been explicitly reported to be heat shock survivors, their capabilities hint toward multiresistance, combined with a high metabolic versatility. The study of paenibacilli from cleanrooms could be beneficial for planetary protection considerations, and further research on this fascinating group of microbes is highly recommended.

In sum it can be stated that extremophilic and extremotolerant microorganisms are present in all spacecraft assembly facilities. Many of them reveal multiresistances and primary producer capabilities. Nevertheless, the information about

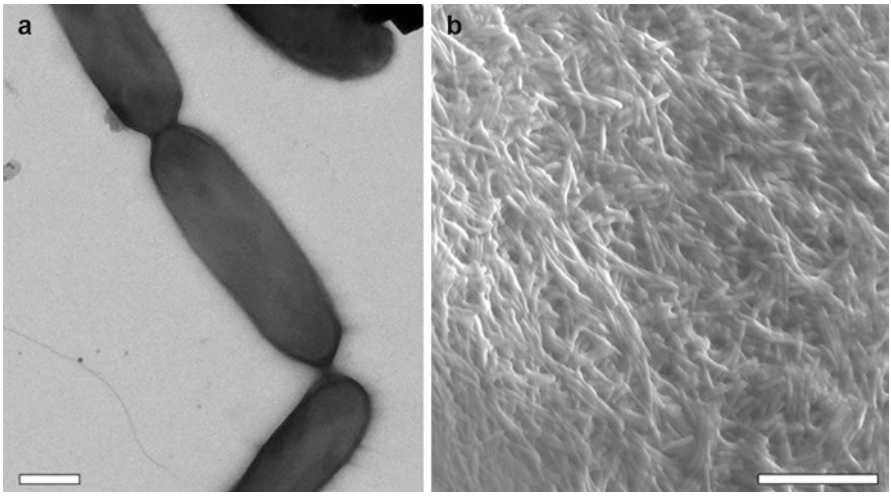

Fig. 10.3 *Paenibacillus cookii* FR1_23. (a) Electron micrograph of a dividing cell; bar, 600 nm. (b) Scanning electron micrograph of a colony; bar, 10 μm

indigenous microbial communities is still very limited: "How many are there?" and "What are they capable of?" are questions that will have to be answered. In order to preserve the integrity of future space travel, the research in the field of planetary protection needs to be enforced further in order to understand the microbial communities in the spacecraft assembly facilities as much as possible.

10.13 The Bacterial Diversity Beyond Cultivation or Cultivation Versus Molecular Analyses

Cultivation as a sole procedure currently does not allow assessing the overall microbial diversity. Previous publications expect a very low percentage (0.1–1 %) of all microbes to be cultivable via standard laboratory techniques (Amann et al. 1995). Nevertheless, our own studies based on the usage of 32 different media and conditions lead to the cultivation of approximately 0.3–5 %, when compared to the quantitative PCR results obtained.

Current molecular microbial diversity methodologies are mainly based on DNA extraction and analysis (mostly depending on the 16S rRNA gene), whereas LAL and ATP measurements have been reported as an acceptable method to obtain insights into the Gram-negative microbial diversity and the ATP content of cleanroom samples. LAL (*Limulus* amoebocyte lysate) analysis is used for the estimation of the Gram-negative, endotoxin-producing bacterial population and measures the presence of lipopolysaccharides. The ATP-based bioluminescence assay can help to obtain insights into the presence and the quantity of viable but non-cultivable cells (Bruckner et al. 2008; La Duc et al. 2007). However, since cells do not contain the same ATP amount (depending on the growth status or the size, Bruckner et al. 2008), quantities can only be estimated.

A strong bias has also been reported for DNA extraction methods and subsequent procedures. Up to now, no extraction method is able to fully extract DNA from spores, without disrupting the nucleic acid. For this reason, DNA-based molecular studies of cleanroom environments detect much more Gram negatives than Gram positives, which are generally harder to lyse or are sporeformers. It can be concluded that many microbes in spacecraft assembly cleanrooms are present as spores, which are not detected by molecular methods but supported by cultivation attempts.

The bias of PCR, still often used to amplify, e.g., the 16S rRNA gene, has also been discussed in several publications, and it is widely accepted that quantitative answers based on standard PCR and subsequent sequencing procedures are rather limited. Furthermore, the selected primers are not universal for the entire microbial group in focus; mispairings can lead to lower PCR efficiency or even to a non-binding of the primers to the target gene. This primer issue is also true for quantitative PCR approaches. Nevertheless, qPCR usually focuses on a very specific microbial group, and the entire methodology is designed for a very effective (up to 100 %) amplification of the target gene. Since measurements are independent other subsequent steps, qPCR allows at least quantitative, comparative predictions.

During the last years, next-generation sequencing methods and also the usage of microarrays ("PhyloChips," La Duc et al. 2009) have been implemented also for cleanroom and other, planetary protection-related studies. Nowadays, molecular, DNA-based methods can even discriminate intact and dead cells and thus allow a more detailed discussion of the results. However, the majority of all detectable microorganisms (99 %) in a cleanroom were found to be dead at the time of sampling (Mahnert et al. 2015; Vaishampayan et al. 2013b).

Molecular high-throughput techniques allow obtaining much more data in a much shorter time and are much more sensitive than cultivation assays. However, even based on metagenomes, we are not able to answer the question whether a microbe or a microbial community is extremophilic or not or could withstand extreme conditions during space flight and thus pose a risk for planetary protection. This information however is crucial for future efforts. It is therefore advisable to put still much effort into novel cultivation strategies (different from standard procedures) in order to increase the percentage of cultivable microorganisms from spacecraft assembly cleanrooms and until the shortcomings of molecular methods are solved.

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