Extremophiles in spacecraft assembly clean rooms

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1 Introduction

1.1 Planetary protection

Over the past decades, the search for life beyond Earth has become one of the greatest motivations for mankind to travel to space. Mars, in particular, being one of the most fascinating planets in our solar system in reach of modern spacecraft, could deliver answers to many open questions. Nevertheless, travel to and landing on Mars is challenging for life detection missions. Since Mars could possibly provide biotopes for its own or terrestrial life, a contamination via microbial hitchhikers from Earth could have severe consequences. The scientific field of planetary protection is concerned about a possible contamination of extraterrestrial environments by terrestrial biomolecules and life forms. Additionally, 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; Anonymous 1967). This scope of ESA's and NASA's planetary protection policies 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.

According to the recommendations of COSPAR (Committee of Space Research), space missions are divided into 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 1) (Anonymous 2002, amended 2005).

Landing missions on Mars are generally assigned to category IV with subcategories a, b, and c. Although Mars is very cold and most likely too dry for life,

H. Stan-Lotter et al. (eds.), *Adaption of Microbial Life to Environmental Extremes*

Table 1. Planetary protection mission categories

Category	Mission type	Possible targets
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 (clean room assembly).	Jupiter, Saturn, Uranus, comets
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 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, and 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 biobuden measurements, (partial) sterilization of hardware, agressive 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

some "special regions" could support life from Earth or indigenous Martian life, should it exist (Rummel 2009). These special regions are locations that might allow the formation and maintenance of liquid water on or under the surface of Mars. Missions that are intended to land in such a region are to be placed under strictest microbial control and limitations. 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 forms of life. Additionally, although not very likely, organisms from Earth could possibly proliferate and contaminate Martian biotopes, competing with potential indigenous life.

Therefore, complete sterility of a spacecraft is a desirable goal. Nowadays, sensitive instruments and detectors onboard do not allow to heat-sterilize the entire spacecraft as was done with the Viking landers 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 clean rooms for integration of precleaned and (as far as possible) presterilized spacecraft hardware.

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

1.2 Clean rooms

In order not to affect or even confound future life detection missions on celestial bodies, which are of interest for their chemical and biological evolution, all spacecraft are constructed in so-called clean rooms and are subject to severe cleaning processes and microbiological controls before launch (Crawford 2005). Clean rooms are certified according to ISO14644-1. Therefore, the clean room class ISO 5 corresponds to the former clean room class 100 (US FED STD 209E), allowing a maximum of 3.5 particles with a maximum 0.5 µm diameter per liter of air.

During assembly, test, and launch operations (ATLOs) of e.g. Mars landers, appropriate cleanliness and sterility levels must be guaranteed: The proper maintenance of the clean room includes a repeated cleaning with antimicrobial agents, particulates are filtered from the air using HEPA (high efficiency particulate air filter) filtering, and even staff working in the clean room must take appropriate actions to minimize any particulate and microbial contamination. Clean room personnel must follow specific access procedures (air locks and 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.

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 includes also the requirement of (daily) sampling of the spacecraft and hardware using swabs and wipes. 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 (Anonymous 1999). The current NASA standard is based on the methods originally developed for the Viking missions in the 1970s. In brief, the surface of a spacecraft is either swabbed (with cotton swabs) or wiped (for larger surfaces). The sampling tools are extracted in water by a combination of vortexing/shaking and sonication. After a heat shock (80°C, 15 min) the suspension is pour plated using trypticase soy agar (TSA) 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. Standard cotton swabs as used for the current

NASA procedure reveal some problems with handling and residues (Probst et al. 2010b); therefore, NASA and ESA are developing novel protocols for the detection of biocontamination.

ESA's new standard methodology is based on the usage of the nylon-flocked swab. Additionally, for better cultivation results of low-nutrient adapted clean room microorganisms, the cultivation medium is R2A instead of TSA, as given in the new ESA standard protocol (Anonymous 2008; Probst et al. 2010b). The recommended sampling size for swabs is only 25 cm^2, whereas polyester wipes are used for the sampling of larger surfaces. If surfaces of space hardware are contaminated above the accepted levels, biocleaning is necessary: alcohols (isopropyl alcohol), disinfectants, and UV exposure are some methods applied to reduce existing contaminants. Furthermore, bioshields can be used to enclose certain (clean) hardware, or the entire spacecraft to avoid contamination (Debus 2006).

In case of Mars, limits on bioburden are based on requirements first imposed on the Viking missions. Therefore, the acceptable microbial contamination is set to about 3×10^5 bacterial spores per Mars landing spacecraft or 300 spores per m^2 on exposed surfaces (Viking presterilization biological burden levels; Anonymous 2002; 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 approximately 20.6 spores per m^2 and therefore within the acceptable range (Pillinger et al. 2006).

1.4 Clean room microbiology

Examination protocols for assembly of spacecraft in clean rooms focus on the detection and enumeration of culturable mesophilic and heterotrophic organisms. Nevertheless, clean rooms are unique environments for microbes: due to low-nutrient levels (oligotrophic), desiccated 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 extremophilic microbial communities (Crawford 2005). For space missions, it is crucial to control the contaminating bioburden as much as possible. 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 clean rooms.

The low biomass is generally problematic, since the sampling and recovery methods themselves are biased and characterized by significant losses during the procedure (Probst et al. 2010b). Furthermore, it is estimated that only 0.1–1% of all microbes present in any biotope can be cultivated using standard cultivation

techniques (Amann et al. 1995), thus increasing the unseen microbial diversity in clean rooms significantly.

Information about the microbial diversity in clean rooms associated with space mission and spacecraft is quite sparse and only a few NASA and ESA supported reports have been published thus far (e.g., Puleo et al. 1977; Venkateswaran et al. 2001; La Duc et al. 2007; Stieglmeier et al. 2009). The first article about the microbial analyses of the two Viking spacecrafts reported that about 7000 samples were taken from both spacecraft surfaces during prelaunch activities in order to determine the cultivable microbial load (Puleo et al. 1977). Besides human-associated bacteria (pathogens and 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 spore-formers has been confirmed in subsequent publications (e.g., Moissl et al. 2007), whereas the portion of *Bacillus* e.g., *Micrococcus* was reported to be significant. In general, 85% of all isolated microorganisms of the NASA JPL group were identified as Gram-positive bacteria (Newcombe et al. 2005).

All of these cultivated microorganisms were obtained from isolation attempts on heterotrophic rich media. However, the chances of surviving space flights are higher for organisms that can thrive under more extreme conditions (Stieglmeier et al.

Table 2. Sampling locations and specifics

	Friedrichshafen 1 (FR1)	Friedrichshafen 2 (FR2)	ESTEC (ES)	Kourou (KO)
Location[a] (site, city)	EADS[b], Friedrichshafen	EADS[b], Friedrichshafen	ESTEC[c], Noordwijk	CSG[d], Kourou
Country	Germany	Germany	The Netherlands	French Guiana
Sampling date	April 2007	November 2007	March 2008	April 2009
Clean room facility	Hall 6, room 6101-04	Hall 6, room 6101-04	Hall Hydra	BAF[e]
Clean room specifics	ISO 5[f]	ISO 5	ISO 8	ISO 8
Sampled surfaces[a]	Various clean room surfaces, e.g., floor, stairs, door knobs; spacecraft	Various clean room surfaces, e.g., floor, stairs; spacecraft	Various clean room surfaces, mainly floor; spacecraft	Various clean room surfaces, mainly floor; floor of Ariane5 container

[a]Further details are given in Stieglmeier et al. (2009) and Moissl-Eichinger (2010)
[b]European Aeronautic Defense and Space Company
[c]European Space Research and Technology Centre
[d]Centre spatial guyanais
[e]Final assembly building
[f]Clean room was nominally operated at ISO 5 but opened to ISO 8 section just before sampling

Table 3. Assortment of media used for the enrichment of microbes from clean rooms

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

[a]Liquid media are shown in italics
[b]*Ae* aerobic and *ma* microaerophilic (<3% O_2)
[c]The extreme conditions which were applied and are discussed in this chapter. If not given otherwise, the incubation temperature was 32°C
[d]Medium recipe and preparation provided in Stieglmeier et al. (2009)

2009). Nevertheless, the search for and successful isolation of microorganisms capable of growing also under more extreme conditions has been reported sparsely (La Duc et al. 2007; Stieglmeier et al. 2009).

In preparation for the recently approved ESA ExoMars mission and also for future lander and Mars Sample Return (MSR) missions, knowledge of the biological contamination in spacecraft assembly facilities, integration, testing, and launch facilities is absolutely necessary. In a very recent separate study of two European and one South American spacecraft assembly, clean rooms were analyzed regarding their microbial diversity, using standard procedures, alternative cultivation approaches, and molecular methods with the aim to shed light on the presence of microorganisms relevant for planetary protection. For this study, the Herschel Space Observatory (launched in May 2009) and the clean rooms used for housing it were sampled during ATLO activities at three different locations (Table 2). Although the Herschel Space Observatory did not demand planetary protection requirements, all clean rooms were under full operation (strict particulate and molecular contamination control) when sampled.

The cultivation procedures and media are summarized in Table 3 (see also Stieglmeier et al. 2009).

In the following chapters, results obtained by the author from cultivation attempts focusing on the extremophilic microbial community in spacecraft assembly clean rooms shall be compared to previously obtained results from US American studies.

1.5 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 clean room itself is an extreme environment hosting mainly extremotolerant microorganisms that accept the extreme circumstances, but prefer moderate conditions for growth. In the following, the author will (for simplifying the terminology) not distinguish between real extremophiles and extremotolerants: for example, the term alkaliphiles concerning clean room isolates include also microorganisms that tolerate, but do not require, alkaline conditions.

2 Spore-forming microorganisms

2.1 Background

Spores are the resting states of bacteria and are usually formed when the organism recognizes a lack in nutrients (C or N source). Endospores of *Bacillus subtilis* are

highly resistant to inactivation by environmental stresses, such as biocidal agents, toxic chemicals, desiccation, pressure, temperature extremes, higher doses of UV, and ionizing radiation (Nicholson et al. 2000; Nicholson and Schuerger 2005). They possess thick layers of coating proteins, and even their DNA is protected by small acid-soluble spore 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).

Germination of spores generally needs an activator, e.g., moderate heat. In culture, amino acids such as alanine seem to support the germination process. In total, almost 20 bacterial genera are able to form spores, but only *Bacillus* and *Clostridium* spores have been subjected to deeper characterization studies (Fig. 1).

The multiresistance properties of such spore-forming microorganisms make them ideal candidates for the survival of space flight. Additionally, commonly applied sterilization conditions such as 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, they have become the main focus of attention in the past decades.

Although 99.9% of all *B. subtilis* spores were killed when exposed to a few minutes of simulated Martian surface conditions (in terms of UV irradiation, pressure, gas

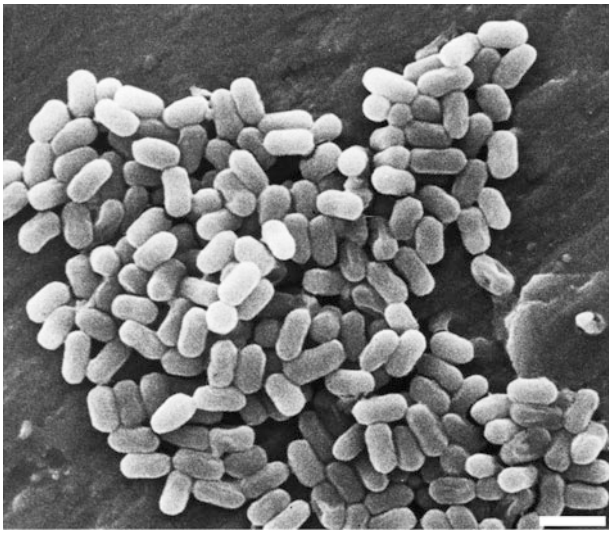

Fig. 1. Scanning electron micrograph of *Bacillus* spores on stainless steel. Bar, 2 µm

composition, and temperature), it has also been shown that dried spores were resistant to UV inactivation when mixed with Mars surrogate soil (Crawford et al. 2003; Schuerger 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 can therefore 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 which are 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 procedure for measurement of biocontamination. Besides the effect that most vegetative cells are killed at 80°C, this heat step is also helpful in stimulating *Bacillus* spores to germinate. Newcombe et al. (2005) reported, that members of the genus *Bacillus* were the predominant microbes among the heat shock survivors, but the isolation of heat-shock-resistant *Staphylococcus*, *Planococcus*, and *Micrococcus* has also been described (Venkateswaran et al. 2001).

For the sake of completeness it shall 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 clean rooms is very limited.

It can be concluded that the current standard procedures of space agencies would not cover the broadest diversity of extremotolerant and spore-forming microbes but provide approximate numbers and estimations as a working basis.

2.2 Results

Bacillus is a typical spore-forming contaminant in spacecraft assembly clean rooms. Puleo et al. (1977) reported already the detection of more than 14 different *Bacillus* strains on the Viking spacecraft. Additionally, *Actinomycetes* and yeast have been detected, but were not characterized further.

Newer studies from spacecraft assembly clean rooms confirmed the presence or even predominance of spore-forming bacteria in cultivation assays based on rich heterotrophic media. Six different *Bacillus* strains have been detected on the Mars Odyssey spacecraft (La Duc et al. 2003), some of them revealing resistance against 0.5 Mrad γ-radiation, 5% H_2O_2 (60 min exposure) or higher doses of UV. In another study, further spore-forming organisms, such as *Sporosarcina*, *Paenibacillus*, *Actinomycetes*, and *Aureobasidium* have been detected (La Duc et al. 2004).

In our recent study of three clean rooms, 32 different culture media were used to target a wide range of different microorganisms (see Table 3). With this approach, the presence of a broader variety of spore-forming microorganisms in spacecraft

assembly clean rooms was revealed. *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 5–25% of all microbes obtained via cultivation. The lowest percentage of spore-formers was found during the second sampling at EADS in Friedrichshafen (FR2). During that time, the clean room was operated at ISO 5 resulting in a higher percentage of human-associated microorganisms and a lower percentage of spore-formers. Most of the spore-forming bacteria observed are associated with environmental biotopes (such as soil) and therefore most likely introduced on items moved into the clean rooms or attached to humans and clothes. It can be concluded that the higher the operational cleanliness of a facility the less spore-forming microorganisms can be expected.

Since almost nothing is known about the resistance of spores obtained from spore-formers other than that of *Bacillus* and *Clostridium*, further analyses of our spore-forming isolates are definitely deemed appropriate.

Bacillus pumilus SAFR-032, an isolate originally obtained from a class 100K (ISO 8) clean room at the Jet Propulsion Laboratory spacecraft assembly facility (JPL-SAF) was reported to form spores with extraordinary UV resistance, outcompeting even a standard dosimetric strain of *B. subtilis* (Newcombe et al. 2005). Different strains of *B. pumilus* have very frequently been isolated from US American spacecraft assembly clean rooms (Puleo et al. 1977) and many of them were described to possess amazing resistances against H_2O_2 (Kempf et al. 2005) or UV light (Link et al. 2004; Newcombe et al. 2005). Generally, these isolates revealed a higher resistance to UV irradiation than the type strain *B. pumilus* (Newcombe et al. 2005).

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

3 Oligotrophic microorganisms

3.1 Background

Oligotrophic (or oligophilic microorganisms) are microbes that are adapted to low-nutrient conditions. Standard laboratory media are usually rich heterotrophic

media providing a broad variety of carbon sources and other nutrients. In contrast, most of the microbes thriving in natural biotopes have to grapple with restriction of nutrients and competition with other organisms. Similarly, clean rooms 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 which are present either have to retreat into a resting state (such as spores) or have to adapt their metabolism to the extreme circumstances. To date, NASA's standard procedures recommend the usage of TSA medium for the cultivation of microbial biocontamination in spacecraft assembly facilities. However, a pharmaceutical clean room study revealed that the portion of cultivables from a clean room production unit could be increased by two orders of magnitude when a low-nutrient medium was applied instead of a rich medium (Nagarkar et al. 2001).

Additionally, when 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.

3.2 Results

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

In our study of European and South American clean rooms, 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, R2A medium was applied even in a 1:10 and 1:100 dilution, respectively. Interestingly, a broad variety of bacteria was cultured on R2A medium diluted 1:100, including *Acinetobacter*, *Balneimonas*, *Brevundimonas*, *Citrobacter*, *Kocuria*, *Microbacterium*, *Micrococcus*, *Moraxella*, *Paenibacillus*, *Sanguibacter*, *Staphylococcus*, *Stenotrophomonas*, and *Streptomyces*, and also including the two spore-formers *Paenibacillus* and *Streptomyces*.

Interestingly, isolates from the first sampling at EADS in Friedrichshafen that were grown on R2A medium diluted 1:10 and 1:100, exceeded the number of cultivables on other (nutrient-rich) media (2.8×10^4 and 4.0×10^4 oligotrophic cultivables per m^2 clean room surface). In comparison, the samples from the other clean rooms revealed a high number of oligotrophs present. Since our approach of

searching for oligotrophs is the first in this field, further studies in other clean rooms will be necessary and are highly recommended.

Based on own observations and results from other studies, the future ESA standard for measurement of biocontamination will rely on R2A medium instead of TSA for the cultivation of spacecraft assembly related microorganisms and will also recommend the usage of even more diluted medium for the growth of oligotrophs as an additional assay.

4 Alkaliphiles and acidophiles

4.1 Background

Although the pH of Mars' regolith was estimated to be about neutral (7.2 ± 0.1; Plumb et al. 1993), the presence of alkaliphiles and acidophiles in spacecraft assembly clean rooms could deliver valuable information for considerations in planetary protection and in particular for clean room maintenance: Most of the disinfectants and detergents used for (bio)cleaning in such clean rooms are either pH neutral or alkaline. It is unclear if thorough 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 clean rooms was examined in two independent studies.

4.2 Results

Samples from diverse spacecraft assembly clean rooms were plated on R2A medium with a pH of 3, 9, and 10.6, respectively (La Duc et al. 2007), or with a pH of 3, 5, 9, and 11, respectively (this project). A broad variety of bacteria tolerating pH 10.6 was reported from US American clean rooms (La Duc et al. 2007) and our studies revealed also various alkaliphiles (growing at a pH of 9 or 11; Table 4). Interestingly, *Bacillus*, *Staphylococcus*, *Brevundimonas*, *Micrococcus*, and *Pseudomonas* were detected in both studies, growing in a medium of pH 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 (see Sect. 1.4) 4.6×10^2 (from the second sampling at Friedrichshafen, ISO 5; see Table 2) up to 8.4×10^3 (at ESTEC, ISO 8; Table 2) alkaliphilic microorganisms per m^2 were measured, whereas the number of alkaliphiles in the samples from Kourou (see Table 2) could not be determined, due to overgrowth of the agar plates.

The detection of acidotolerants was much more difficult. The colony counts on agar plates with medium of pH 5 were very low; no isolate was obtained during the Herschel campaign tolerating pH 3, whereas an acidotolerant colony

Table 4. Extremophilic isolates[a] from global spacecraft assembly clean rooms

Genus	Oligotrohs[b]	Psychrophiles[c]	Alkaliphiles[d]	Anaerobes	Thermophiles[e]	Halophiles[f]	CO_2 fix	N_2 fix	Species; in brackets: location of isolate[g]; type of extremophile[h]
Acinetobacter	○	○							*A.* sp., *A. ursingii* (FR2)
Actinotalea			○						*A. fermentans* (KO)
Aerococcus				○		○			*A. urinaeequi* (KO)
Arsenicicoccus				○			◆		*A. bolidensis* (FR2)
Arthrobacter			○	○				◆	*A.* sp. (KSC/alk, KO/an, N_2)
Bacillus			○ ○ ○ ○	○ ○ ○ ○	○ ○ ○	○			*B. thermoamylovorans* (ES/alk, FR1/an), *B. gibsonii* (FR2/alk), *B. licheniformis* (FR1/alk, ES/an,therm), *B. pumilus* (KO/an, JSC/alk), *B.* tc (FR2, ES, KO/an), *B. badius* (KO/therm), *B. coagulans* (ES, KSC/therm), *B. megaterium* (KO/halo), *B.* sp. (JSC/alk)
Balneimonas	○		○						*B.* sp. (KO)
Brachybacterium			○						*B. paraconglomeratum* (LMA)
Brevibacillus			○		○				*B. agri* (FR1)
Brevibacterium			○						*B. frigoritolerans* (ES)
Brevundimonas	○		○ ○						*B. nasdae* (FR2, KO), *B. diminuta* (KSC)
Cellulomonas				○ ○			◆		*C. hominis* (FR1, KO/an, KO/aut)
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)
Enterococcus				○ ○			◆		*E. casseliflavus* (KO/aut), *E. faecalis* (ES/an), *E. faecium* (ES/an)
Facklamia			○	○					*F.* sp. (KO)

(*continued*)

Table 4 (*continued*)

Genus	Oligotrohs[b]	Psychrophiles[c]	Alkaliphiles[d]	Anaerobes	Thermophiles[e]	Halophiles[f]	CO_2 fix	N_2 fix	Species; in brackets: location of isolate[g]; type of extremophile[h]
Geobacillus					○ ○ ○ ○				*G. caldoxylosilyticus* (ES, KSC), *G. stearothermophilus* (JPL), *G. kaustophilus* (JPL), *G. thermodenitrificans* (JSC)
Georgenia				○					*G. muralis* (KO)
Kocuria	○		○						*K. rhizophila* (ES), *K. rosea* (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)
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)
Oceanobacillus			○						*O.* sp. (JPL)
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)
Propionibacterium				○ ○ ○					*P. acnes* (FR1, FR2, ES), *P. avidum* (ES)
Pseudomonas		○	○ ○					◆	*P. luteola* (FR2/N_2), *P. xanthomarina* (KO/alk,psy), *P. stutzeri* (KSC/alk)
Roseomonas		○	○						*R. aquatica* (KO)
Sanguibacter	○			○			◆	◆	*S. marinus* (KO)
Sphingomonas			○						*S. oligophenolica* (JSC), *S. trueperi* (JSC)

(*continued*)

Table 4 (*continued*)

Genus	Oligotrohs[b]	Psychrophiles[c]	Alkaliphiles[d]	Anaerobes	Thermophiles[e]	Halophiles[f]	CO_2 fix	N_2 fix	Species; in brackets: location of isolate[g]; type of extremophile[h]
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)
Streptomyces	○		○					◆	*S. luteogriseus* (ES)
Tessaracoccus				○					*T. flavescens* (KO)

[a]*Circles* and *diamonds* indicate each locations, where microbes were detected; each symbol represents one sampling location. *Black*: Isolates from this study (see Table 2 for abbreviations of locations). *Red*: Isolates from US American studies (La Duc et al. 2007)
[b]Oligotrophs were grown on R2A medium diluted 1:100
[c]Psychrophiles were grown on R2A medium at 4°C
[d]Alkaliphiles were grown on R2A medium of pH 10.6 or 11, respectively
[e]Thermophiles were grown at 50°C, 60°C or 65°C, respectively
[f]Halophiles were grown on R2A medium containing 10% NaCl
[g]Locations: KSC (Kennedy Space Center), JSC (Johnson Space Center), LMA (Lockheed Martin Aeronautics), and JPL (Jet Propulsion Laboratory)
[h]*an* anaerobic, *alk* alkaliphilic, *oligo* oligotrophic, *therm* thermophilic, *halo* halophilic, *aut* autotrophic, N_2 N_2 fixing, *psy* psychrophilic, and *B. tc Bacillus thuringiensis/cereus* goup

count was reported only for the US Lockheed Martin Aeronautics Multiple Testing Facility (LMA-MTF) and Kennedy Space Center Payload Hazardous Servicing Facility (KSC-PHSF) facilities (La Duc et al. 2007). All other samplings were negative for acidotolerants. None of the isolates obtained during the US American study was analyzed further or revealed multiresistant properties (La Duc et al. 2007).

A significant preference of alkaline media by bacteria was found in all facilities analyzed so far. One isolate obtained by the group of Venkateswaran was a salt and alkalitolerant bacterium, which was described very recently as *Bacillus canaveralius* (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 outpacing of acidophiles or nonalkalitolerants. If so, the selectivity via the pH of cleaning agents could be circumvented by using detergents with alternating pH.

5 Autotrophic and nitrogen fixing microorganisms

5.1 Background

The capability to fix nitrogen from the gaseous atmosphere or to grow autotrophically on CO_2 are important properties 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 clean rooms and also the Martian environment are depleted in organic materials, primary producers, if present, could pave the way for secondary settlers.

Previous US studies have not reported any attempt to cultivate primary producers. All studies looking for cultivables from spacecraft and associated clean rooms have used heterotrophic, solidified rich media (except Stieglmeier et al. 2009; see below). The assortment of special media used in this study also included media selective for chemolithoautotrophs, using CO_2 as the only carbon source. Media providing nitrogen only in the gas phase were also applied.

5.2 Results

Seven isolated bacterial genera were able to fix CO_2 and eight were able to fix N_2. Further details about these bacterial genera are given in Table 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 obtained from coastal sediment (Fujian province of China), and none of the observed properties had been reported in the original strain description (Huang et al. 2005). It can be imagined that the clean room isolate shows distinct properties due to the adaptation to the extreme biotope, or that 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*).

6 Anaerobes

6.1 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 soils, aerobic desert soils, or other biotopes, such as the human body (Tally et al. 1975; Peters and Conrad 1995; Küsel et al. 1999). In the latter case, they may become potential contaminants of spacecraft assembly facilities by staff, who is in close contact with 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 their reproduction and metabolism and can even be inhibited or killed by oxygen.

So far, not much is known about the presence of anaerobically growing microorganisms in spacecraft clean rooms. The presence of anaerobic microorganisms, which were enriched using the BD GasPaK system, in surface samples from US clean rooms has rarely been reported. Members of the facultatively anaerobic genera *Paenibacillus* and *Staphylococcus* have been isolated in the course of a study of extremotolerant microorganisms (La Duc et al. 2007).

A proper anaerobic cultivation necessitates the application of methods like 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 anaerobic cultivation technology and a broad variety of microbes capable of anaerobic growth was isolated (Stieglmeier et al. 2009).

6.2 Results

A variety of anaerobic microorganisms was successfully isolated from all four clean room samplings. In total, 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 4 and Fig. 2).

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

Only a comparatively low percentage of microbes grew strictly anaerobic (*Propionibacterium*, *Corynebacterium*, *Desulfotomaculum*, and *Clostridium*; 0.7–4% of all isolates at each location). *Propionibacterium acnes*, typically found on human

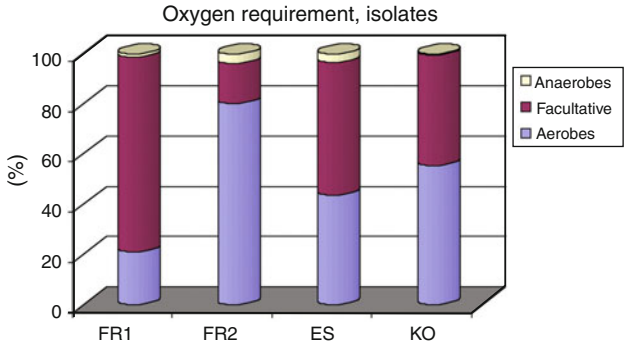

Fig. 2. Oxygen requirement of isolates. Numbers represent the percentage retrieved. Physiological capabilities are based on either own experiments or published data (for isolates grown on aerobic media only)

skin, was isolated from each European clean room and therefore the most prominent strict anaerobe detected. All strict anaerobes except *Desulfotomaculum* were opportunistic pathogens and isolated from rich, 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 did not produce black colonies, which would have indicated a sulfate reducing activity. As it was clarified in a previous publication, *D. 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 spore-former. Other spore-forming microorganisms capable of anaerobic growth were *Bacillus*, *Paenibacillus*, and *Clostridium*. Further details are given in Table 4.

These data were confirmed by a very recent analysis of the anaerobic microbial diversity in NASA's clean rooms at the Jet Propulsion Laboratory. This study was based on a microbial enrichment of clean room samples under anaerobic conditions, which was subsequently analyzed via cultivation, 16S rRNA gene sequence analysis and microarrays (Probst et al. 2010a). *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. 2010a).

Our results indicate that the facultatively and strictly anaerobic microbial community is quite diverse and may even be dominant in spacecraft assembly clean rooms.

7 Thermophiles and psychrophiles

7.1 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 are much below 0°C. Actually, also Earth's biosphere is quite cold – more than 70% of its freshwater 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 long-term 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 exhibit "primordial" metabolic capabilities (for instance chemolithoautotrophy). Thermophiles are also generally considered more resistant than moderate or cold-loving microorganisms.

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

7.2 Results

A study of US American spacecraft assembly clean rooms did not report any growth of microorganisms on R2A medium following incubation for 10 days at 4°C (La Duc et al. 2007). Our study was selective for microorganisms capable of growing at 10°C and 4°C, respectively. The duration of incubation 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, was the only isolate not able to grow at 32°C, the standard cultivation temperature.

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

The selective enrichment of thermophiles on R2A medium at 65°C (La Duc et al. 2007) or 60°C and 50°C, respectively (this study), allowed the isolation of three *Geobacillus* and one *Bacillus* strain (La Duc et al. 2007) and *Bacillus*,

Brevibacillus, *Paenibacillus*, and *Geobacillus* (this study). Interestingly, *Bacillus coagulans* and *Geobacillus caldoxylosilyticus* were isolated in both studies, whereas the latter was described as an obligate thermophile (*Saccharococcus caldoxylosilyticus*; see Ahmad et al. 2000), which is in accordance to the observations made in this study (no growth was observed at 32°C). It is unclear, how *Geobacillus* (spores) entered the clean room (although they would not be capable to proliferate under the thermal conditions of a clean room) and why this organism was detected in two independent studies in clean rooms 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 the spores possibly survived strict cleanliness control conditions after being carried into the facilities by humans and materials.

8 Halophiles

8.1 Background

Halophiles have been discussed as possible survivors on Mars, since the Martian liquid water (if available) would 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 clean rooms. In order to obtain insights into the distribution of these organisms in clean rooms samples from two studies were plated on R2A medium containing different concentrations of NaCl.

8.2 Results

Samples from US American clean rooms were plated onto R2A medium containing 25% (w/v) NaCl, but no growth was observed (La Duc et al. 2007). In contrast, samples obtained from the Herschel campaign (see Sect. 1.4) were plated on R2A medium containing 3.5% and 10% (w/v) NaCl, respectively. Growth on R2A medium 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 4). The most prevalent species tolerating 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% NaCl.

Despite the obvious presence of halophilic/halotolerant microbes, *B. megaterium* was the only spore-forming isolate that was detected on salty agar plates.

9 Archaea

9.1 Background

Archaea, the third domain of life, were considered for more than 20 years as extremophiles that were ecologically restricted and highly adapted to specific and often hostile biotopes. Meanwhile Archaea have been detected in almost any "normal" biotope such as marine and freshwater or soil (Bintrim et al. 1997; Karner et al. 2001; Rudolph et al. 2004). Many of the extremophilic Archaea have very interesting properties. In the eyes of many researchers they are "primitive" in their metabolism, which actually means that they can act as primary producers. This property could be an advantage when settling in new biotopes. Detailed experiments with vegetative (hyper-)thermophilic Archaea have revealed unexpectedly high tolerances against desiccation, vacuum, and UV or gamma radiation (Beblo et al. 2009). It is unclear however, whether these organisms could withstand the extremely harsh conditions during space travel, or lack of nutrients and low temperatures in extraterrestrial environments.

The main procedure to detect microorganisms in clean rooms is still directed toward Bacteria, but Archaea are more and more considered a possible source of biocontamination for spacecraft. The possibility that Archaea, such as methanogens or halobacteria, might be able to survive a space flight and survive or even to thrive on Mars, has already been discussed (Landis 2001; Kendrick and Kral 2006). Nevertheless, the existence of Archaea in human-controlled and rigorously cleaned environments has not been assessed before, so that it was unclear, if Archaea could even be found in spacecraft-associated clean rooms.

The vast majority of mesophilic and psychrophilic Archaea still resists cultivation, and also the attempt to cultivate Archaea from spacecraft assembly facilities failed (Moissl et al. 2008; Moissl-Eichinger 2010). For this reason, the detection of Archaea has to be based on molecular studies only, which are presented in the following section.

9.2 Results

In 2008, we reported the detection of archaeal 16S rRNA gene signatures in two US American spacecraft assembly clean rooms (Moissl et al. 2008). Using a very sensitive PCR approach 30 different cren- and euryarchaeal sequences were derived

from NASA facilities. The omnipresence of Archaea in global clean rooms was confirmed in a very recent study (Moissl-Eichinger 2010). Archaea were detected in all clean rooms analyzed (second sampling Friedrichshafen, ESTEC and Kourou). As already reported in 2008 most of the gene sequences obtained clustered within the Crenarchaeota group 1b (now Thaumarchaeota) (Spang et al. 2010). The closest cultivated neighbor, candidatus *Nitrososphaera gargensis*, shows more than 4% difference in the 16S rRNA gene sequence; other close relatives from various natural biotopes are still uncultivated. Many representatives of this group were described to possess genes for ammonia oxidation (Hatzenpichler et al. 2008). Future studies looking for functional genes such as archaeal *amoA* genes could most likely enlighten the physiological capabilities of the Thaumarchaeota group detected in clean rooms.

The detection of these Thaumarchaeota in different spacecraft assembly clean rooms is significant: Different methods have been used and the studies were performed in two different laboratories. Overall, 48 different crenarchaeal sequences were obtained from five global clean rooms thus far (Moissl-Eichinger 2010). Additionally, molecular 16S rRNA gene sequence analysis data were supported by using fluorescence *in situ* hybridization (FISH) and real-time PCR (qPCR). Hybridization signals were obtained from samples taken in the clean room Hydra at ESTEC: rod shaped microbes reacted positively with Archaea-specific probes. QPCR revealed that the average number of Archaea per m^2 clean room surface amounted to around 2×10^4, approximately two to three logs lower than the estimated total number of Bacteria. Although the number of Archaea appears quite low, their presence was as consistent as typical bacterial clean room contaminants. Interestingly, most of the Bacteria detected are human commensals or opportunistic pathogens (e.g., *Staphylococcus*; Moissl et al. 2007; Stieglmeier et al. 2009). This finding could possibly hint toward a linkage of Archaea and humans, who might be carriers also for this group of microorganisms.

Besides Thaumarchaeota, methanogens and a halophilic archaeon were also (sporadically) detected (Moissl et al. 2008; Moissl-Eichinger 2010).

Our work has shown, that Archaea are an omnipresent, significant part of the microbial community in spacecraft assembly clean rooms all over the world. Since most of the data are based only on molecular analysis, it is unclear whether these Archaea could pose a threat with regard to planetary protection aspects. Further studies will be necessary and additional attempts for cultivation are recommended.

10 Other extremophiles

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 resistance against radiation (UV and gamma) as well as against hydrogen peroxide has been in the focus of interest, since these techniques are usually applied for the sterilization of spacecraft components (besides dry heat).

La Duc et al. (2007) reported the presence of microorganisms which were resistant to UV-C ($1000\,J\,m^{-2}$) and 5% hydrogen peroxide. These resistances were found separately in spore-forming microbes only (*Bacillus*, *Nocardioides*, and *Paenibacillus*), whereas *B. pumilus* was resistant against both treatments. The detection of a hydrogen-peroxide-resistant *B. pumilus* has been reported even earlier (Kempf et al. 2005).

The multiresistant *B. pumilus* SAFR-032 was studied extensively at the Jet Propulsion Laboratory. The whole genome has been sequenced and annotated (Gioia et al. 2007). Although the sequence revealed differences to and, in addition, unknown genes compared to closely related species *B. subtilis* or *B. licheniformis*, the *B. pumilus* genome seems to lack genes functioning in resistance to UV or H_2O_2, which were found in other *Bacillus* strains. Further studies will certainly be necessary to understand the molecular basis of the 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 medium. The entire study focused on microorganisms, and the conditions would certainly 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 further research is highly recommended. Nevertheless, the detection of *Aureobasidium pullulans* from spacecraft assembly facilities was reported, a yeast-like fungus surviving 1 Mrad gamma radiation for 5.5 h (Bruckner et al. 2008).

11 Lessons learned from the Herschel campaign: 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 4). Besides that, we have shown, that many microbes thriving or surviving in clean rooms are able to fix nitrogen and/or carbon dioxide, and could therefore serve as primary producers. The following list summarizes the main findings of our recent study:

- Spore-formers were present in each facility analyzed, but the total number did not exceed 25% of all isolates. The lowest percentage was seen in FR2, which was the "cleanest" environment sampled.

- The highest cell counts were obtained on media with lower nutrient contents and higher pH values, hinting toward a possible influence of environmental selective forces.
- Primary producers were found in unexpected diversity: autotrophs and N_2 fixing microbes were successully isolated.
- Strictly anaerobic bacteria were cultured from each facility, but are present in a low number only (up to 4%), although facultatively anaerobic microbes were found to add up to 78% of isolates.
- Thermophiles, psychrophiles, and halophiles were found in the clean rooms.
- Archaea were detected in each facility and their presence is significant. The properties of the (cren-)archaeal clean room community are unclear, but Archaea are able to persist and intact cells seem to be present.
- Cleanliness level of a clean room definitely influences the microbial diversity. The broadest diversity of cultivables was seen in Kourou and ESTEC samples (both clean rooms were operated at ISO 8). It can be assumed that also the environmental conditions have an influence on the microbial diversity within the clean rooms, 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 typical environmental organisms.

Furthermore, we have shown that not only *Bacillus* but also other (spore-forming) bacteria can play a significant role in clean room environments. For instance, several *Paenibacillus* strains have been detected and at least three of

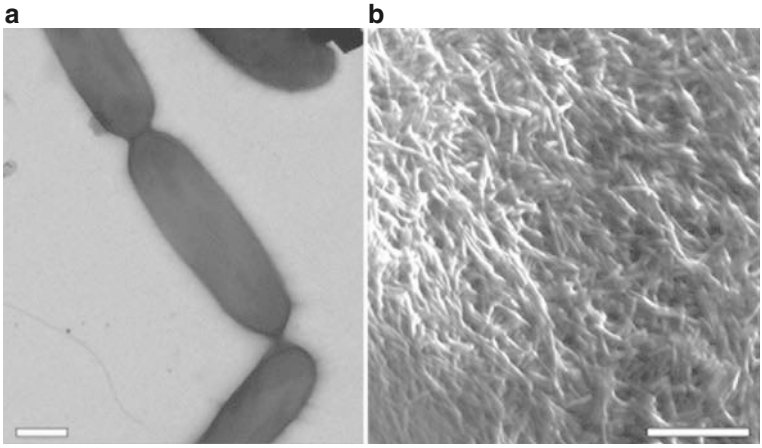

Fig. 3. *Paenibacillus cookii* FR1_23. (**a**) Electron micrograph of a dividing cell; bar, 600 nm. (**b**) Scanning electron micrograph of a colony; bar, 10 μm

them were identified to be novel isolates. Many bacilli are dependent on complex organic compounds for their metabolism. Interestingly, although closely related to bacilli, some paenibacilli seem to have the ability of nitrogen and carbon dioxide fixation. Initial resistance experiments with our isolates revealed a pronounced capability to survive desiccation, vacuum, heating, or Mars-cycle simulations (Fig. 3).

One novel isolate (ES_MS17, *Paenibacillus purispatii* sp. nov.) was shown to have profound metabolic capabilities for nitrogen conversion processes (Behrendt et al. 2010). Although paenibacilli have not explicitly been reported to be heat shock survivors, their capabilities hint toward multiresistance, combined with a high metabolic versatility. The study of paenibacilli from clean rooms could be beneficial for planetary protection considerations, and further research on this fascinating group of microbes is highly recommended.

In summary it can be stated that extremophilic and extremotolerant microorganisms are present in all spacecraft assembly facilities. Many of them reveal multiple resistances and capabilities of primary producers. Nevertheless, the information about indigenous microbial communities is still very limited: "How much is 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, research in the field of planetary protection needs to be enforced further with the aim to understand the microbial communities in the spacecraft assembly facilities as much as possible.

12 The bacterial diversity beyond cultivation, or cultivation vs. molecular analyses

Cultivation as a singular procedure currently does not allow assessing the overall microbial diversity. Previous publications predicted 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 led to the cultivation of approximately 0.3–5%, when compared to the qPCR results obtained (data unpublished).

Current molecular methodologies for assessing microbial diversity are mainly based on DNA extraction and subsequent PCR analysis (mostly 16S rRNA gene sequences), whereas LAL (limulus amoebocyte lysate) and ATP measurements have been reported as acceptable methods to obtain insights into the Gram-negative microbial diversity and the ATP content of clean room samples, respectively. LAL 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 nonculturable cells (La Duc et al. 2007; Bruckner et al.

2008). However, since cells do not contain the same amounts of ATP (which depends on the growth status or the size; Bruckner et al. 2008), quantitative measurements are strongly biased.

A strong bias has also been reported due to extraction methods for DNA and PCR. 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 clean room environments detect much more Gram negatives than Gram positives; the latter are generally harder to lyse or are spore-formers. It can be concluded that many microbes in spacecraft assembly clean rooms are present as spores, which are not detected by molecular methods, but are identified by cultivation attempts.

The bias of PCR has also been discussed in several publications and it is widely accepted, that no quantitative answers can be given based on standard PCR and subsequent cloning procedures (e.g., von Wintzingerode et al. 1997). Furthermore, selected primers are not universal for the entire microbial group in focus; mispairings can lead to lower PCR efficiency or even to a nonbinding of the primers to the target gene (Huber et al. 2002). This primer issue is also true for quantitative PCR approaches. Nevertheless, qPCR usually focuses on a 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 from cloning or other subsequent steps, qPCR allows at least quantitative, comparative predictions.

Compared to cultivation, molecular methods also detect dead cells that have influence on planetary protection issues only as a possible source of contamination with biomolecules. On the other hand, the sensitivity of PCR allows observing signatures of outnumbered and uncultivated inhabitants. Like previous studies based on standard 16S rRNA gene cloning, molecular studies of clean room environments revealed an unexpected broad diversity of microbes: The presence of diverse Bacteria and even Archaea has been shown only via molecular analyses (Moissl et al. 2007, 2008).

The knowledge of the microbial diversity in spacecraft assembly clean rooms has significantly increased with the recent studies (Bruckner et al. 2008). The methodologies to obtain insights into the qualitative (who is there?) microbial diversity are proceeding fast, but the overall (quantitative) microbial diversity of clean rooms (how much is there?) is still quite unclear. The next crucial step is the preparation of a typical model community, containing all main (and most resistant) microbes that were detected in spacecraft assembly clean rooms. With this artificial community the detection methods can be tested and more insights into quantitative information from molecular studies be gained (Kwan et al. 2011).

Although cultivation-independent methodologies are advancing fast, cultivation of microbes is still essential to investigate their abilities and resistances. Molecular techniques allow obtaining much more data in a much shorter time, and high-

throughput methods such as microarrays and whole genome sequencing (454 sequencing and other technologies) are fascinating. A very recent study has reported the successful usage of microarrays (PhyloChips) also in the field of planetary protection (La Duc et al. 2009). Nevertheless, none of these techniques allows insights into the metabolic capabilities and resistance properties of microbes. Novel microarray techniques, such as the "GeoChip," searching for specific metabolic genes (He et al. 2007) could give stronger insights, but will not completely answer the open questions about resistance and properties. This information, however, is crucial for future planetary protection aspects. It is therefore advisable to put much effort into novel cultivation strategies (which are different from standard procedures) in order to increase the percentage of cultivables from spacecraft assembly clean rooms. Microbial analyses of biocontamination risks have to be as broad as possible and different technologies will be necessary to obtain the most complete picture on the specific microbial community in spacecraft assembly clean rooms.

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