Diversity and resistance of microorganisms in a European spacecraft testing clean room

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Diversity and resistance of microorganisms in a European spacecraft testing clean room

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I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale.

Marie Curie (1867-1934)

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This is dedicated to the two of you…

Abbreviations

°C	degree celsius
AB	antibiotic
Amp	ampicillin
Approx.	approximately
ATP	adenosine 5'-triphosphate
ATV	automated transfer vehicle
BC	biocide
BCIP	5-Brom-4-chlor-3-indolyl-phosphat
bidest	bidistilled
bp	base pairs
Caltech	California Institute of Technology
CC	area inside the clean room (controlled)
CC+	area inside the ATV, inside the clean room (highly controlled)
Cfu	colony forming unit
DLR	Germany Aerospace Center
DMSO	Dimethylsulfoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
DSMZ	German Collection of Microorganisms and Cell Cultures
E.	Escherichia
e.g.	for example (exempli gratia)
EDTA	ethylenediamine tetraacetic acid
EHF	ESTEC HYDRA facility
ESA	European Space Agency
ESTEC	European Space Research and Technology Centre
et al.	and others (*et alii*)
Fig.	Figure
g	gram
h	hour(s)
HM	heavy metal
HYDRA	Hydraulic Shaker Facility
i.e.	that is (id est)

IN	inducer
IPTG	isopropyl-β-D-thiogalactopyranosid
ISO	International Organization for Standardization
ISS	International space station
J	joule
kHz	Kilohertz
NASA	National Aeronautics and Space Administration
NCBI	National Center for Biotechnology Information
p.a.	pro analysi
PBS	Phosphate Buffered Saline
RODAC	Replicate Organism Direct Agar Contact
STEDV	standard deviation
TNTC	too numerous to count
Tris	tris(hydroxymethyl)aminomethane
UC	area outside the clean room (uncontrolled)
v/v	volume per volume
w/v	weight per volume

I. Introduction

The term biology was first introduced at the start of the 19th century by Karl Friedrich Burdach and Gottfried Reinhold Treviranus and is formed by combining the Greek βίος (bios), meaning "life", and λόγος (logos), meaning "study".

However man's study of life started much earlier. While humans were unknowingly using microbes for thousands of years (e.g. the use of yeast for bread & wine), one of the major breakthroughs for biologists was the development of the microscope in the early 16th century, making it possible to view and study features and organisms which were too small to be seen by the naked eye. This achieve-ment opened the scientists' view and their minds to life in the micro-range. Since then the modern science of microbiology has advanced and diversified immensely allowing a more complex and in depth view on life. Scientist even today are still surprised by the ability of prokaryotes to survive or even thrive under extreme conditions, until then, thought to be uninhabitable. Prokaryotes are central members of earth's biota providing important functions inside a working ecosystem. Their role ranges from catalyzing processes like decomposition (recycling nutrients) to the fixation of nitrogen (into "usable form" for plants) or the production of oxygen by *cyanobacteria*. The estimated numbers of prokaryotic organisms present on this planet amounts to 4-6 x 10^{30} cells, effectively making them the richest and widest spread life-form of this planet (Whitman *et al.,* 1998). Prokaryotes are most abundant in the open ocean, in soil, and in oceanic and terrestrial subsurfaces where, for example, up to 4 x 10^{7} cells representing 2-18 x 10^{3} different species can be found in one gram of soil (Torsvik *et al.*, 2002). Though small as the prokaryotes are, by their sheer number they can affect the environment on geological levels (Croal *et al.*, 2004).

1.1 Planetary Protection

The concept of Planetary Protection (PP) encompasses the responsibility and commitment of space faring nations to prevent the biological contamination of Earth and other planets during our exploration of the solar system (Rummel and Meyer, 1996; Rummel, 2000). The ability of micro-organisms to survive under a multitude of extreme conditions and endure even in the vacuum of space (Horneck, 1981) stresses the importance of analysing outgoing and incoming spacecraft for unwanted contamination by such organisms.

The international rules were finally implemented in 1967 with the U.N. Outer Space Treaty which was signed by the United States, the Soviet Union, and the United Kingdom, and then ratified by the Secretary General of the United Nations (COSPAR 2002; Outer space treaty, 1967; Sterns and Tennen, 1995). Since then PP-considerations have become part of every planning- and, if needed, execution-phase for extraterrestrial missions (Sweetser *et al.*, 1995).

Five distinct PP categories and thereby bioburden (spores per m^2) restriction levels were established to correlate the imposed limits with the scientific interest in the visited planet and the nature of the undertaking (e.g. flyby or landing) [Table 01]. The PP category IV was additionally subdivided into three distinct classes to differentiate between areas of special interest on the visited planet i.e. Mars areas with a high/low probability of containing water.

Table 01: Planetary Protection mission categories

Interest of planet visited	Type of mission	Mission category
Not of direct interest for understanding the process of chemical evolution. No protection of such planets is warranted (no requirements).	Any	I
Of significant interest relative to the process of chemical evolution, but only a remote chance that contamination by spacecraft could jeopardize future exploration.	Any	II
Of significant interest relative to the process, chemical evolution and/or the origin of life or for which scientific opinion provides a significant chance of contamination which could jeopardize, a future biological experiment.	Flyby, orbiter	III
	Lander, probe	IV
	Earth-return (all solar bodies)	V

Depending on the mission the PP requirements can be met in two ways: for orbiter or flyby missions a comprehensive analysis of the flight path and orbit stability can be calculated. If the possibility of an accidental contamination of the visited planet can be kept below a 5% chance for the next 20-50 years (NASA, 2005) the PP requirements are deemed fulfilled. Otherwise, or for missions including a lander or probe, the mandatory bioburden as stated by the PP rules must be met before the mission can lift off. For example, missions to regions on Mars where life is thought to be possible, the overall bioburden of the complete surface of the landing space craft may not exceed 30 verifiable spores before start (NASA, 2005).

Though the PP rules are discussed and partly revised every four years by an international group of experts during the Committee on Space Research (COSPAR) meeting, the contamination survey protocols, established in the 1970's for NASA's Viking mars missions (Puleo, 1977), are still in use.

To comply with the PP requirements strict anti-contamination procedures and controls need to be established during the space hardware assembly phase. As seen with Europe's "Beagle 2 Mission" specialized sterilization methods needed to be established. In addition, the craft's assembly in specialized, dedicated clean rooms was needed to reduce the bioburden below the level specified by the PP rules (Pillinger *et al.*, 2006).

1.2 Clean rooms

Clean rooms are designed to provide an almost contamination-free workspace for highly sensitive fields of work including hospital operation theatres, electronic precision parts, pharmaceutical production or the assembly of space hardware (DeVincenzi *et al.*, 1996; Moeller, 1992; Tweedie, 2005). The environment inside a clean room is continuously controlled to restrict the number of pollutants i.e. aerosol particles, chemical vapours or microbes inside the facility. For most industrial clean rooms the implied requirements include the properly filtration of the room's air (HEPA-Filter) and special garments worn by the work crew. For more specialised areas these restrictions can increase drastically (Pillinger *et al.*, 2006). Depending on the applied standardization system and the cleanliness level met by the facility, clean rooms are divided into 9 (ISO), respectively 7 (FED-STD) categories [Table 2].

Table 02: Specifications of particle numbers per m^3 of different clean rooms classes for the two established standards (FED-STD and ISO)

FED-STD 209E	ISO 14644-1	0.1 µm	0.2 µm	0.3 µm	0.5 µm	1 µm	5 µm
	ISO 1	10	2				
	ISO 2	100	24	10	4		
1	**ISO 3**	1,000	237	102	35	8	
10	**ISO 4**	10,000	2,370	1,020	352	83	
100	**ISO 5**	100,000	23,700	10,200	3,520	832	29
1.000	**ISO 6**	1,000000	237,000	102,000	35,200	8,320	293
10.000	**ISO 7**				352,000	83,200	2,930
100.000	**ISO 8**				3,520000	832,000	29,300
	ISO 9				35,200000	8,320000	293,000

In most cases for industrial clean rooms the number of particles per cubic foot represents the main controlled parameter. However, during medicine production or the assemble of space probes destined to land on other planets of our solar system, the number of microorganisms present within the facility is additionally highly restricted (DeVincenzi *et al.*, 1996; Jimenez, 2001). At present a number of established techniques exist to assess the level of microbial surface contamination like contact plates, swab-rinse, swabbing and heap filter suction. The swab-rinse technique was introduced by Mannheimer and Ybanez in 1917 (Mannheimer and Ybanez, 1917) and in 1944 became one of the standard methods recommended by the American Public Health Association for contamination monitoring of food utensils and sanitations (Tiedeman, 1944). Techniques like vacuum probe and contact plates were established sometime later and depending on the area of work and the environment tested, different approaches became standard procedures. For surveying the microbial contamination of space hardware NASA tested several methods in the late 1960's. Though the direct surface contact technique (RODAC plates (Replicate Organism Direct Agar Contact)) (Baldock, 1974) has the advantage of creating actual representations of the spatial distribution of the present

microorganisms, the method can only be applied effectively on flat surfaces and will leave a residue on the tested object (Angelotti *et al.*, 1964). This technique cannot be applied for monitoring complex electronic parts or space flight hardware due to these limitations (Kirschner and Puelo, 1979). In the 1970's protocols for several sampling techniques (e.g. wipe-rinse or swabbing) were established (Favero, 1971; Puleo *et al.*, 1967; Puleo *et al.*, 1973) and constituted by NASA during the Viking Mars Lander missions (Puleo *et al.*, 1977). Today these techniques are still in use for assessing the microbial contamination of space hardware assembling facilities (NASA, 2005).

1.3 Phylogenetic analysis of the environmental bacterial diversity

Traditionally the identification of the environmental bacterial diversity was solely based on cultivation dependent microbiological methods. The isolated bacteria were subjected to several tests identifying an array of morphological, physiological and biochemical classification features (On and Holmes, 1991). In the 1970's the concept of polyphasic taxonomy was termed by Colwell (Colwell, 1970) and aimed at the integration of several generally accepted classification criteria into a consensus type of taxonomy (Vandamme *et al.*, 1996). The tested features encompassed several kinds of information (phenotype, genotype, phylogenetic) to delineate the taxa at all levels (Murray *et al.*, 1990). This cultivation based approach is, so far, the only option to obtain reliable phenotypic properties of bacterial strains (Vandamme *et al.*, 1996; Wayne *et al.*, 1987).

We know today that the sole dependency on culture based methods for community studies introduces a strong bias and exhibits several drawbacks. Cultivation of a heterogeneous bacterial sample is difficult since any used medium or cultivation condition exerts an intrinsic selection pressure due to its specific composition and properties (Amann *et al.*, 1995). Therefore, every time an environmental sample is cultivated a part of the present bacterial community will be favoured by the applied conditions and dominate the culture. Ultimately, once a cultivation step is included into the test setup, the number of different species identifiable will only be a fraction of the actual present bacterial diversity (Kaeberlein *et al.*, 2002; Rodriguez-Valera, 2004). The difference between the microscopic bacterial count and the actual number of colonies grown during cultivation was named by Staley and Konopka in 1985 the "great plate count anomaly" (Staley and Konopka, 1985) and still cannot be fully compensated today. Depending on the sample site, and using standard laboratory procedures, only 0.001% to 15% [Table 03] of the actual bacterial community can be cultivated (Amann *et al.*, 1995).

Table 03: Culturability as a percentage of cultivable bacteria in comparison to total cell counts (from: Amann *et al.*, 1995)

Habitat	Culturability
Seawater	0.001-0.1%
Freshwater	0.25%
Mesotrophic lake	0.1-1%
Unpolluted estuarine waters	0.1-3%
Activated sludge	1-15%
Sediments	0.25%
Soil	0.3%

In 1965 Zuckerhandl and Pauling were the first to suggest that cellular molecules could be used as tracers for evolutionary progression, but almost another two decades elapsed before Carl Woese used ribosomal RNA sequences to establish a phylogenetic tree (Woese, 1987). Even though there are other useable marker genes like those of heatshock proteins, ATPases or Topoisomerases (Marsh, 1999) the small subunit ribosome gene (16S rDNA) became the most used taxonomic bacterial marker gene. Today databases like the Genbank project of the "National Center for Biotechnology Information" (NCBI, www.ncbi.nlm.nih.gov) has almost four hundred thousand 16S sequences from over 1,700 bacterial geniuses encompassing 12,351 species (June 2007). The 16S rDNA gene is an ideal candidate for a marker of phylogenetic relatedness since it combines several key features which are necessary to be able to use the gene as a "molecular clock". The ribosome is an ancient well conserved molecule, which is essential in the central processes of translating DNA stored information into proteins: the protein synthesis (Madigan *et al.*, 2003). Therefore, this molecule is ubiquitously present in all pro- and eukaryotic organisms (Alberts *et al.*, 2002; Madigan *et al.*, 2003). The 1.5kb long gene features highly variable to strongly conserved regions and, thereby, offers the possibility to discern and classify the phylogenetic relationship of very close to very distinct organisms (Amann *et al.*, 1995; Embley and Stackebrandt, 1994). Finally, there is no evidence for lateral 16S rDNA gene transfer between bacterial species, whereby the insights gained by sequence comparisons can be assumed to be true predictions about the evolutionary relationship of these organisms (Pace, 1997). The start- and end-region of the gene are highly conserved across all bacteria and archaeal domains offering the opportunity to amplify almost all the 16S rDNA gene by PCR. This setup is the basis for the cultivation-independent approach of environmental community studies (Giovannoni *et al.*, 1990). As mentioned above, at present more than three hundred eighty thousand 16S sequences are known and the classification of newly isolated sequences by base comparison has become a standard practise. Once the method was established new insights into the evolutionary history were made fast; and organisms were, for the first time, grouped together due to their genetic relationship and not by morphologic similarity. Since the beginning when Woese proposed 12 cultivable bacterial phyla (Woese, 1987), 14 new cultivable phyla and 26 candidate phyla, which

include not yet cultivated species, were identified (Hugenholtz *et al.*, 1998a; Rappe and Giovannoni, 2003). Overall, the fact that today half of the known bacterial phyla cannot be cultured in the laboratory stresses the importance of including culture independent methods for the description of bacterial communities.

1.4 Pitfalls of 16S rDNA phylogenetic analysis

Though the use of cultivation independent phylogenetic analysis of complex bacterial communities has become more and more common (Head *et al.*, 1998), this kind of techniques has its own pitfalls and biases (DeLong and Pace, 2001; von Wintzingerode, 1997). The "set up" of these techniques must be strictly controlled since broad range 16S rDNA PCR is a highly sensitive method and the chance of false positive results due to contamination of the working equipment needs to be addressed carefully. Laboratory reagents like PCR enzymes (*Taq*), plastic articles (e.g. Eppendorf tubes) or DNA extraction kits can be contaminated with trace amounts of bacterial DNA (Grahn *et al.*, 2003; Schmidt *et al.*, 1991; van der Zee *et al.*, 2002) and therefore need to be controlled extensively before use.

Another disadvantage of molecular-based methods for bacterial community studies lies within the samples themselves. Environmental samples contain a high bacterial diversity where the relative quantity of single species (and by that rDNA sequences) can diverge over orders of magnitude. In addition, it is known, that the copy number of the *rrn* operons (N = 1 to 13) can vary between bacteria (Coenye and Vandamme, 2003) and that minor variations (N = 2 to 3) can even be observed inside the same species (Candela *et al.*, 2004). Though it is theoretically possible to detect rare species by amplifying genes from the few original copies, the binding of the primers is not an actively guided process and, thereby, heavily influenced by the starting copy numbers of each 16S rDNA sequence (Farrelly *et al.*, 1995). These variations will lead to a biased amplification and an inaccurate identification of the present bacterial species.

The second disadvantage lies within the PCR technique itself. The standard universal primers used to amplify all bacterial 16S rDNA do not exhibit "equal universal" properties. Depending on the species 16S rDNA sequence, these universal primers will bind with different affinities to their DNA target (Forney *et al.*, 2004). Therefore, in samples with multiple 16S rDNA's, a taxa bias will be introduced during amplification due to the dissimilar affinity of the primers to the heterogeneous DNA sequences (Baker *et al.*, 2003; Horz *et al.*, 2005). Even though several methods were established to minimize the bias of the amplification step, like the use of multiple primer pairs, denatured primers (Baker *et al.*, 2003; Polz and Cavanaugh, 1998; Suzuki and Giovannoni, 1996; von Wintzingerode *et al.*, 1997), so far no totally unbiased DNA amplification with universal primers has been published.

1.5 Bacterial abilities and resistance

The ability of bacteria to adapt and withstand impairing conditions and substances can on the one hand be beneficial for mankind, but on the other hand can lead to severe problems. When focusing on the ability of bacteria to survive the harmful effect of antibiotics used to cure infections, this ability poses a major threat to human health. In the last years, the fact that a rapid increasing number of bacterial species have became resistant to standard antibiotic treatments became a key international health challenge and has attracted the attention of many scientists (Struelens *et al.*, 2004; Wright, 2007). Whereas bacterial resistance to antibiotics poses a major problem, the ability of bacteria to survive in highly contaminated (Turpeinen *et al.*, 2004) or even radioactive environments (Zavilgelsky *et al.*, 1998) led to a new field of applied science: bioremediation (Urgun-Demirtas, *et al.* 2006). Several publications describe the attempt to develop bacterial hybrids which can grow in strongly polluted surroundings. These bacteria are further able to bind or metabolically degrade the present toxic components, effectively cleaning up the environment while they are growing (Hirata *et al.*, 2005; Malik, 2004). The capability of the microbial community to interact and change their surroundings is another example for the two-sided coin these abilities represent. While microbial leaching is increasingly used (Olson *et al.*, 2003) to gather raw materials like i.e. copper or uranium from the environment, a related kind of interaction, the bacterial process of biocorrosion (Beech *et al.*, 2005), leads to the deterioration of i.e. metals, plastics or concrete and creates a yearly damage of billions of dollars.

The fact that bacteria exhibit a highly flexible and transferable genetic basis (D'Costa *et al.*, 2006), coupled with a doubling time of as low as 20 minutes leads to this remarkable adaptability to surrounding conditions.

So far two concepts for the acquisition of resistances are known today: vertical and horizontal evolution.

Vertical evolution is driven by spontaneous mutation (mutation rate for most bacterial genes is approximately 10^{-8}) and natural selection (Denamur and Matic, 2006). The change of even one base can lead to a total resistance against the compound in question. A well studied example for this kind of process is the ability of bacteria to survive high doses of Rifamp after one specific base is changed in the Polymerase beta-subunit (Jin and Gross, 1988).

Horizontal evolution is the acquisition of (resistance) genes from other organisms.

Three routes are known by which bacteria can exchange genes: conjugation, transduction and transformation. *Conjugation* involves cell-to-cell contact where DNA crosses a sex pilus from donor to recipient. During *transduction*, a virus transfers the genes between mating bacteria.

Transformation describes the direct acquisition of DNA from the environment. The DNA may be present due to the prior lysis of other bacteria or because it was released from another cell.
Several species of the genus *Streptomyces* exhibit an inherent resistance against their own antibiotic: streptomycin. The genes encoding this resistance can be transferred between species within one habitat (e.g. bacterial soil community). Once these genes are incorporated into the genome of the recipient bacterial strain the streptomycin resistance is effectively passed on to a new species (Wiener *et al.*, 1998).

For some bacteria another possibility to survive harsh conditions is the transformation of viable cells into sturdy, durable inactive forms: spores. One of the closely studied bacteria which are able to switch to a sporeform is *Bacillus subtilis*. The first mentioning of the organism can be dated back to 1835 when Mr. Christian Gottfried Ehrenberg described a circumflexed rod (*Vibrio subtilis)* which can survive even the harshest conditions. In 1872 the organism was renamed to the form still used: *Bacillus subtilis* by Ferdinand Julius Cohn. As endospore, *Bacillus subtilis* can survive heat (>100°C), radiation, many chemicals (i.e. acids, bases, alcohol, chloroform) and long periods of desiccation. The mechanisms that account for this resistance include the impermeability of the endospore coat, the dehydration of the cytoplasm and the production of special proteins that protect the spores DNA (Setlow, 2006).
Even though the change into a dormant and resilient state cannot be considered an active form of resistance, it is an effective adaptation to survive i.e. extreme seasonal or harsh climatic changes and is used by various bacteria (Nicholson *et al.*, 2000). Especially in the light of the strong probability that spores can survive interstellar travel between i.e. Earth and Mars (Mileikowsky *et al.*, 2000) this kind of “passive resistance” is crucial for PP considerations.

Intention of this work:

The goal of this study was to analyse and improve the standard biological contamination survey of clean rooms used for the assembly of space hardware by the European Space Agency (ESA). Once the integration of modern, molecular based biological techniques into the survey setup was achieved a European space associated clean room was to be sampled as a model system to test the newly established protocols. The cultivable as well as uncultivable phylogenetic composition of the facility was to be determined by 16S rDNA analysis to establish a comprehensive overview of the present bacterial community. The so isolated cultivable bacterial species should then be characterized further to achieve a more in depth understanding of the functional properties of a clean room bacterial community.

II. Materials and methods

2.1 Used organisms, plasmids and oligonucleotides

The organisms used in this study are listed in Table 04. The plasmid pDrive (*oriEc*(pUC), Plac*lacZ*, Kan^r, Amp^r) from Qiagen (Hilden, Germany) was used for all cloning approaches applied in this study. Table 05 provides an overview of the primers utilized for PCR amplification reactions.

Table 04: Used bacterial strains

Bacterial Strain	Characteristics	Reference
Bacillus subtilis 168 (DSM 402)	type strain	DSMZ
QIAGEN EZ competent cells	[F'::Tn*10*(Tc^r) *proA*$^+$*B*$^+$ *lacI*^{q}ZΔ*M15*] *recA1 end A1 hsdR17* (r_{K12}^- m_{K12}^+) *lac glnV44 thi-1 gyrA96 relA1*	Qiagen (Hilden, Germany)

Table 05: Used oligonucleotides

Name	**Sequence**	**Reference**
27F	5'-GAG TTT GAT CMT GGC TCA G-3'	Lane, 1991
1492R	5'-CGG YTA CCT TGT TAC GAC-3'	Kane *et al.*, 1993
T7	5'-AAC AGC TAT GAC CAT G-3'	pDrive manual (Qiagen)
SP6	5'-CAT TTA GGT GAC ACT ATA G-3'	pDrive manual (Qiagen)

Y= C oder T; M= A oder C

2.2 Chemicals, media and reagents

Chemicals, media and reagents which were used for microbial work were either autoclaved for 20 min at 121°C or sterile filtrated before use to avoid contamination.

If not specifically mentioned otherwise, the media were obtained from Becton, Dickinson and Company, Sparks, USA. To prepare solid media for petri dishes, 1.5% of agar/agar (Becton, Dickinson and Company, Sparks, USA) was added to the media in question. Only materials of purity "p.a" and distilled water were used for the preparation of media and buffer. The composition specifications refer to a final volume of 1 liter.

2.2.1 Buffers

2.2.1.1 Phosphate buffered Saline (PBS)

7.0 g	Na_2HPO_4
3.0 g	KH_2PO_4
4.0 g	NaCl
1000 ml	$H_2O_{bidest.}$

The pH was adjusted to 7.0 before autoclaving.

2.2.1.2 Tris-Acetate-EDTA Buffer (TAE-Buffer) 10x

48.46 g	Tris (0.4 M)
3.72 g	EDTA-Na_2-salt (0.01 M)
12.01 g	Acetic acid (0.2 M)
1000 ml	$H_2O_{bidest.}$

The pH was adjusted to 8.0 before autoclaving.

2.2.2 Rich media

2.2.2.1 LB (Luria Bertani) Medium

10.0 g	Bacto™ Tryptone
5.0 g	Bacto™ Yeast Extract
10.0 g	Natriumchlorid (NaCl)
1000 ml	$H_2O_{bidest.}$

The pH was adjusted to 7.0 before autoclaving.

2.2.2.2 R2A Medium

0.5 g	Bacto™ Yeast Extract
0.5 g	Proteose Peptone (Difco no. 3)
0.5 g	Casamino acids
0.5 g	Glucose
0.5 g	Soluble starch
0.5 g	K_2HPO_4
0.5 g	$MgSO_4$ x 7 H_2O
15.0 g	Agar
1000 ml	$H_2O_{bidest.}$

The pH was adjusted to 7.2 before autoclaving.

2.2.2.3 Tryptone Soy Medium (TSB-medium)

30.0 g	BBL™ Trypticase™ Soy Broth
3.0 g	Bacto™ Yeast Extract
1000 ml	$H_2O_{bidest.}$

Depending on the experimental setup the pH was adjusted to either 7.0, 4.4 or 9.2 before autoclaving.

2.2.2.4 Super Optimal Catobolite Repression Medium (SOC-medium)

2%	Tryptone
0.5%	Yeast Extract
10 mM	NaCl
2.5 mM	KCl
10 mM	$MgCl_2$
10 mM	$MgSO_4$
20 mM	Glucose

2.2.3 Antibiotics, heavy metals, biocides and media additives

The reagents and media additives [Table 06] were prepared as stock solutions and sterile filtrated. The stocks were either frozen at -20°C until use, or kept at 4°C for a maximum of three weeks. Antibiotics and other heat sensitive additives were added to the media after it was autoclaved and cooled down to ca. 55°C.

Table 06: Concentrations of used antibiotics, heavy metals, biocides and other media additives

Reagent	Description	Solvent	Stock solution (x/ml)	Working solution (x/ml)
Arsenic acid (KH_2AsO_4)	HM	H_2O_{dest}	240 mM	100/10/1/0.1 mM
Cadmium chloride-hemipentahydrate	HM	H_2O_{dest}	240 mM	100/10/1/0.1 mM
Ciprofloxacin	AB	H_2O_{dest}	240 µg	100/10/1/0.1 µg
Cobalt(II) chloride-hexahydrate	HM	H_2O_{dest}	240 mM	100/10/1/0.1 mM
Copper(II) chloride	HM	H_2O_{dest}	240 mM	100/10/1/0.1 mM
Formaldehyde	BC	H_2O_{dest}	16% (v/v)	8% (v/v)
Glutardialdehyde	BC	H_2O_{dest}	2% (v/v)	1% (v/v)
Gramicidin	AB	50% Ethanol	240 µg	100/10/1/0.1 µg
IPTG	IN	H_2O_{dest}	100 mM	50 µM
Kanamycin	AB	H_2O_{dest}	20 mg	100 µg
Mercury chloride	HM	50% Ethanol	24 mM	10/1/0.1/0.01 mM
Nisin	AB	H_2O_{dest}	240 µg	100/10/1/0.1 µg
Penicillin G	AB	H_2O_{dest}	240 µg	100/10/1/0.1 µg
Rifampin	AB	50% Ethanol	240 µg	100/10/1/0.1 µg
Trimethoprim	AB	H_2O_{dest}	240 µg	100/10/1/0.1 µg
X-Gal	Dye	Dimethyl-formamid	40 mg	80 µg
Zinc(II) chloride	HM	H_2O_{dest}	240 mM	100/10/1/0.1 mM

AB = Antibiotic, BC = Biocide, HM = Heavy metal, IN = Inducer

2.3 General microbiology methods

2.3.1 Growth conditions and archiving

2.3.1.1 Liquid cultures

Liquid cell cultures were used to grow cells for resistance testing or DNA extraction. To prepare liquid bacterial cultures, an autoclaved toothpick was used to either pick a single colony from an agar plate or to obtain material from the organism's stored cyrostock. The toothpick was then aseptically snapped into test tubes containing 10 ml of liquid TSB or LB-medium and incubated for 20 h at 32°C while shaking (200 rpm). For incubation of bacteria containing plasmids the antibiotic kanamycin (30 µg/ml) was added to the medium to sustain the selective pressure.

2.3.1.2 Plate cultures

Plate cultures were utilized for a) the cultivation of mixed bacterial environmental samples b) creation of pure cultures from mixed samples c) bacterial resistance testing where the cfu count needed to be established and d) selection of positive transformed clones.

The environmental bacteria were plated on petri dishes containing TSB agar (heat tolerant heterotrophic bacteria) or R2A agar (unselected heterotrophic bacteria). LB agar containing petri dishes were used to culture the created 16S rDNA clones. To select for positive transformed clones IPTG (50 µM), X-gal (80 µg/ml) and kanamycin (30 µg/ml) were given to the medium after it cooled down below 50°C.

Plates containing TSB or LB media were cultured at 37°C whereas R2A media containing plates were incubated at 25°C. Environmental samples were cultured for up to seven days whereas 16S rDNA clones were only incubated for 24h.

2.3.1.3 Archive cultures

Cryostocks were created to store the newly isolated organisms from the Hydra facility sampling site. Therefore, 300 µl of a turbid overnight culture [see liquid cultures 2.5.1.1] were mixed with 1.2 ml of autoclaved glycerol (100%) and stored at -80°C. All the resistance tests in this thesis were made with freshly prepared overnight cultures from the organism's cyrostock.

2.3.1.4 Isolation of microorganisms from mixed cultures

Bacteria were isolated from mixed environmental cultures using the streak plate method. Therefore, single, morphological different colonies (size, shape, texture, color, raised, concave, etc.) were picked from each plate with a sterile inoculation loop, and streaked onto a new agar plate. The medium used for the agar plates depended upon the origin of the sample: heat shocked samples were streaked on TSB agar plates and untreated environmental samples on R2A agar plates. The plates were inverted and the bacteria incubated either at 32°C (heat shocked) or 25°C (untreated). During

the incubation period the agar plates were routinely examined for the phenotypic characteristics of the isolated bacterial colonies. If one or more phenotypes were detected on the agar plates, single distinct colonies were picked and once more streaked out onto new plates. This isolation process was repeated until the agar plates featured only a single isolated, phenotype.

2.3.2 Microbiological analysis

2.3.2.1 Colony forming units (cfu) test

The Colony Forming Unit (cfu) test (Puck and Marcus, 1956) was used to determine the survival rate of organisms after treatment with different reagents.

50 µl of the treated bacterial suspensions were plated out in three different suitable 10-fold serial dilutions and incubated for 20 h at 32°C. All plating was done in duplicates. After the incubation period, the formed colonies were counted. The mean survival rate can be calculated by $S = \left(N/N_0\right)$ where N is the titer (cfu) of untreated cells and N_0 is the titer (cfu) of treated cells.

2.3.2.2 Swabbing efficiency test

2.3.2.2.1 Spiking swabs with *Bacillus subtilis* spores

Bacillus subtilis 168 (DSM 402) spores were prepared as described in Moeller *et al.* 2005. Before use, the spore batch was subjected to a heat shock (80°C, 10 min) and stored in twice distilled H_2O. Sterile rayon swab were aseptically removed from their container and 100 µl of a *Bacillus subtilis* suspension containing 10^4 spores/ml were dripped directly onto the heads of the swabs. The swabs were replaced to their container, incubated for 10 min at room temperature (RT) and then processed further.

2.3.2.2.2 Preparation of "pre-contaminated" surface samples

The samples were prepared by spotting 4 x 10² *Bacillus subtilis* 168 (DSM 402) spores on 5 x 5 cm² plates. The plates consisted of different material (aluminum (AL-Mg3 DIN Nr. 3.3536), V2A steel (DIN Nr. 1.4301), Kapton (3M™ Polyimide Film Tape 5413; 3M, USA), Teflon (PTFE) and Multilayer Insulation (MLI) foil (aluminized Kapton)) approved and used in spaceflight. The plates were cleaned using ethanol and subsequently autoclaved to avert possible pre-contamination. To gain a relatively homogenous distribution of the spores 60 drops of 5 µl each were evenly spotted over the area. The plates were dried for 20 h and processed the next day. A control for the total amount of spores spotted was created by pouring 20 ml of warm (42°C) TSB-Agar (Tryptic Soy Broth DIFCO; Becton, Dickinson and Company, USA) on prepared but "unswabbed" plates after the drying period. This control was done for each surface during each experimental set.

2.3.2.2.3 Swabbing the surface samples

Sampling of the surfaces took place as follows: A sterile cotton (P. Boettger OHG, Germany) or rayon swab (MW & E, England) was aseptically removed from its container and moistened with sterile ddH_2O. The swab was angled at 30 degrees to the surface and the area swabbed. This was done thrice, each time changing the direction of the swabbing motion by 90 degrees. Furthermore, the head of the swab was also rotated each time the swabbing motion changed. After sampling, the swabs were replaced to their container and 2 ml of sterile Phosphate Buffered Saline (PBS) was added. The swab was incubated for 10 min at room temperature. During this time, the swab was vortexed three times for 10 sec (Mixer (UZUS10 VTX-3000L), Laboratory & Medical Supplies, Brigachtal, Germany). At the end of the 10 min incubation period, 2 min of sonication at 35kHZ (Qualilab (USR54H); Merck Eurolab, Germany) followed. Finally, the 2 ml of PBS were poured into a sterile petri dish and 20 ml of warm (42°C) TSB-Agar was added and the solutions mixed by gentle swirling. After the agar solidified, the plates were incubated over night at 37°C and the cfu counted.

2.3.2.2.4 Cell lysis of test cultures and environmental samples

The swab-heads together with the remaining supernatant [5.2.2.3 and 5.4.1] were used for the DNA extraction. 500 µl of Qiagen Lysis-Buffer ATL (containing edetic acid and sodium dodecyl sulphate, pH 8.3) was added to the samples and vortexed. This was followed by the addition of 20 µl Proteinase K (20 mg/ml). The samples were incubated for 120 min at 60°C after which 500 µl of Qiagen Lysis-Buffer AL (10 mM Tris·Cl; 0.5 mM EDTA; pH 9.0 (Qiagen, Hilden, Germany)) and 1 µl of carrier RNA was added. An incubation phase at 70°C for 30 min and subsequently freezing step at -60°C for another 30 min followed. The DNA extraction was completed by boiling the samples for 10 min at 95°C. The supernatants were then transferred to sterile Eppendorf tubes. To collect any residual lysate retained in the swab heads the heads were centrifuged in a QIAshredder (Qiagen, Hilden, Germany) for 5 min at 14.000 rpm. The flow-through was pooled with the sample's supernatant and stored at -20°C until further processed.

2.3.3 Sampling at the ESA Hydra facility, Noordwijk (Netherlands)

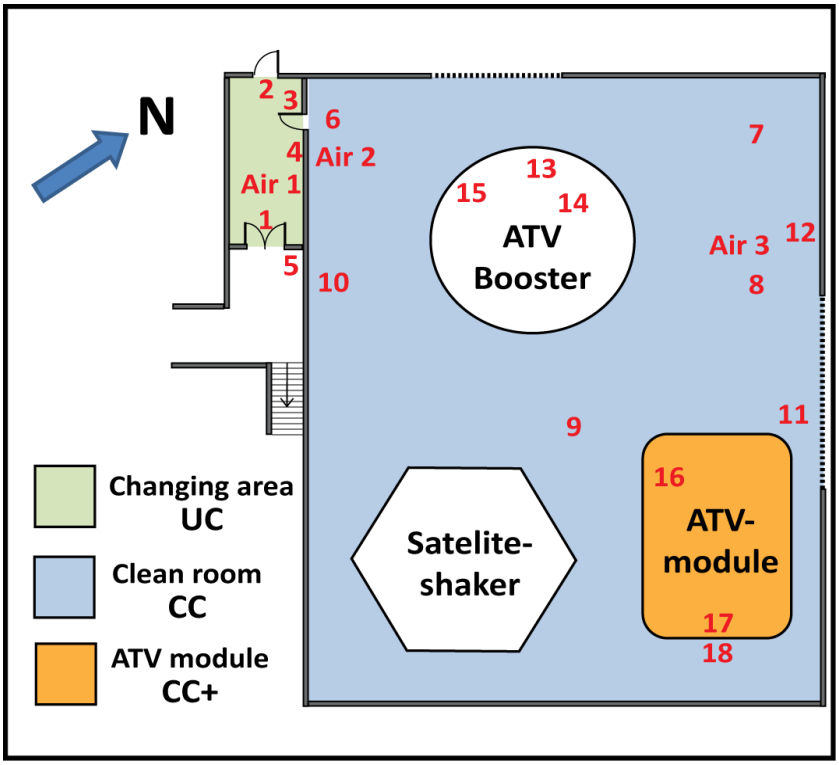

Figure 01: Swab and air sample locations inside ESA's Hydra facility
The three different cleanliness levels present in the ESA facility are indicated by the green (outside the clean room), blue (inside the clean room) and orange (inside the ATV) colors.

2.3.3.1 Sampling setup at the Hydra facility, Noordwijk (Netherlands)

Before the actual sampling procedure took place, 18 sampling locations were chosen to achieve a representative coverage of the different areas and cleanliness levels inside the Hydra facility. Therefore, five locations in the changing area outside the restricted area (uncontrolled = UC), 11 locations inside the class 100k clean room (controlled = CC) and two areas inside the automated transfer vehicle (ATV) (highly controlled = CC+) were selected.

Furthermore, one location outside and two locations inside the classified area were chosen for air sampling (please refer to Figure 01 for the exact sample locations).

An air-conditioned container was used to transport the samples back to the German Aerospace Centre (DLR) keeping the temperature constantly below 10°C. After arrival in the laboratory (ca. 4 h) the samples were directly processed as described in [2.3.3.2-3].

Table 07: Areas swabbed inside ESA's Hydra facility

Sample site	Area	Location	Inclination
EHF 01	Changing room (UC)	Floor in front of 1st outside (entrance) door	horizontal
EHF 02	Changing room (UC)	Floor in front of 2nd outside (entrance) door	horizontal
EHF 03	Changing room (UC)	Floor in front of exit to the clean room	horizontal
EHF 04	Changing room (UC)	Surface of the door that leads to the clean room	vertical
EHF 05	Hallway (UC)	Surface of the shoe cleaner	horizontal
EHF 06	Clean room (CC)	Floor in front of the exit to the changing room	horizontal
EHF 07	Clean room (CC)	Floor in front of the computer area	horizontal
EHF 08	Clean room (CC)	Surface of a working bench	horizontal
EHF 09	Clean room (CC)	Floor in the middle of the clean room	horizontal
EHF 10	Clean room (CC)	Surface of a computer desk in front of keyboard	horizontal
EHF 11	Clean room (CC)	Floor close to the gate to the other clean room	horizontal
EHF 12	Clean room (CC)	Wall next to a working bench (close to #08)	45°
EHF 13	Clean room (CC)	ATV outside structure (ground level)	vertical
EHF 14	Clean room (CC)	ATV support ring (2nd floor)	horizontal
EHF 15	Clean room (CC)	ATV floor of the scaffold (2nd floor)	horizontal
EHF 16	Clean room, inside ATV (CC+)	ATV-ICC inside, racking	vertical
EHF 17	Clean room, inside ATV (CC+)	ATV-ICC inside, close to the exit/entrance to ISS (rear panel)	horizontal
EHF 18	Clean room (CC)	ATV-ICC outside, ring structure	horizontal
EHF Control CC	Clean room (CC)	Control classified	-
EHF Control UC	Changing room (UC)	Control unclassified	-

Figure 02 represents a schematic explaining the sampling-code identifier. This system was used to catalog every cultivable isolate and detected uncultivable species. The sample-code includes: the date and type of sampling, the area the isolate or clone was collected from and (if appropriate) the treatment before the cultivation.

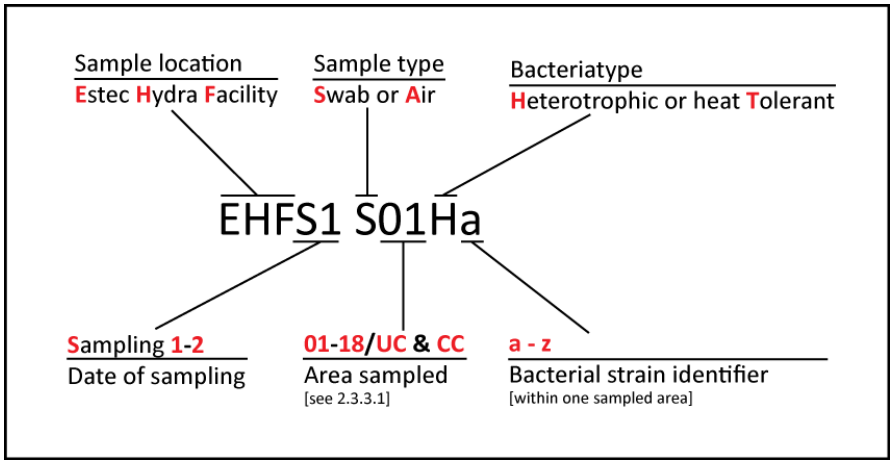

Figure 02: Sample-code schematic

2.3.3.2 Ground sampling of microorganisms at ESA's Hydra facility

In each chosen locations four adjacent, identical squares of 25cm^2 (sample A-D) were swiped using Alpha® swabs (ITW-Texwipe). The swabs were aseptically removed from their container and moistened with sterile, distilled H_2O. Each swab was angled at 30 degrees to the surface and a 5 x 5cm area was wiped. Each square was swapped three times, each time changing the direction of the swabbing motion by 90 degrees. Moreover, every time the swabbing motion changed the head of the swab was rotated. After the procedure was conducted the swab heads were cut off into 2 ml Eppendorf tubes. While the first two samples (sample A & B) were pooled into one Eppendorf tube containing 1.6 ml of Phosphate Buffered Saline (PBS) for direct analysis, the last two swab-heads (sample C & D) were stored as a backup in Eppendorf tubes without the addition of PBS.

2.3.3.3 Air sampling of microorganisms at ESA's Hydra facility

Air samples were taken using a Sartorius AirPort MD8 (Sartorius AG, Goettingen, Germany), which was equipped with gelatine air filters (17528-80-ACD, Satorius, Goettingen, Germany).

At each sampling site two gelatine filters were incubated by drawing 300 l of air through the filters with a speed of 30 l/min. The filters were oriented horizontally at all times during sampling. Afterwards the filters were aseptically placed into sterile petri dishes and transported back to the laboratory. The filters were then, either processed directly, or frozen at -80°C for a later analysis.

2.3.4 Cultivating of collected bacteria from ESA's Hydra facility

2.3.4.1 Processing of surface samples

All samples were processed within five hours after the actual sampling took place.

To detach the organisms from the swabs, the samples were treated as described in the NASA Standard procedures for the microbial examination of space hardware (NPG: 5340.1D, 1980). Therefore, the samples were vortexed for 1 min (Reax 2000, Fa. Heidolph, Kelheim, Germany) which was followed by a 2 min sonification step at 37°C and 35kHZ (Qualilab (USR54H); Merck Eurolab, Germany). A second 1 min vortexing step finished the detaching phase.

2.3.4.2 Surface samples – cultivable aerobic, total heterotrophic bacteria

After the above described detaching treatment, 400 µl of each samples supernatant was aseptically transferred to a new tube and further processed. The samples were 10-fold serial diluted in PBS up to a dilution of 10^{-5}. 100 µl aliquots of each dilution were plated out in duplicates on 9 cm^2 R2A agar plates. The samples were incubated for seven days at 25°C, and the formed colonies (cfu) counted at day two and seven.

2.3.4.3 Surface samples – cultivable aerobic, heat tolerant bacteria

To select for heat tolerant/spore forming microorganisms 800 µl of the undiluted supernatant was subjected to a heat shock (80°C, 10 min). After the treatment, the supernatant was divided into two fractions of 400 µl and portioned into two separate petri dishes. 20 ml of TSB agar, kept fluid at 50°C, was added to the petri dishes. Both fluids were then mixed careful by swirling. The agar plates were incubated for seven days at 32°C and the total aerobic count was enumerated on day two and day seven.

In addition to the above described treatments the swab heads and the remaining supernatant (ca. 400 µl) were kept frozen at -80°C for further DNA analysis.

2.3.4.4 Air samples – cultivable aerobic, total heterotrophic bacteria

For each sample location one of the two gelatine air filters was placed directly on a R2A Agar plate and incubated at 32°C for seven days. The filter was positioned within the petri dish so that the "exposed face" pointed towards the medium. On day two and day seven the grown colonies (cfu's) were counted.

2.4 Resistance testing of cultivable bacterial isolates

2.4.1 Isolate culture preparation for resistance testing

The archived bacteria were picked from frozen Cryostocks [2.5.1.3] and incubated for 20 h in 10 ml 1x TSB medium. After the incubation phase, the turbidity of the cultures was measured and then diluted as described for the different resistance tests [see 2.4.3-7].

2.4.2 Bacterial growth determination (96 well plate)

Bacterial growth was detected by monitoring of the medium turbidity due to presence of grown cells. The turbidity of the wells was quantified using a Wallace Victor II multiplate well reader (Perkin Elmer, Gaithersburg, USA) at an excitation wavelength of 490 nm. The actual cell growth was then calculated as the mean growth of the duplicates and normalized using the untreated, maximal growth of the bacteria. Therefore, it was possible to obtain the percentile growth of the bacteria under the different influencing factors. The lack of growth inhibition, indicating resistance, was then compared among the different isolated cultivable bacteria.

2.4.3 Antibiotic, heavy metal and biocide resistance testing (96 well plate)

The 96 well plates (Bio-one PS Microplate (650101), Greiner BioChemica, Flacht, Germany) were prepared one day in advance and incubated overnight at 37°C to screen for possible contamination during the plate preparation phase. In each well 100 µl of the appropriate 2x stock solution of the antibiotic/heavy metal/biocide [Table 06] were added to 100 µl of 2x TSB medium (please refer to Figure 03 for exact 96 well setup). An aluminum adhesive seal (ABgene Part #AB-0626) was used to avoid evaporation during the overnight incubation phase. The plates were checked the next morning for contamination, condensate was removed by centrifugation (1000 rpm, 3 min) and the foil re-placed by an air permeable lid. Of the 1:10 diluted overnight culture 40 µl were pipetted into each well. All samples were tested in duplicates. The plate was then incubated for 20 h at 37°C. Background-controls were established by adding 40 µl PBS instead of cells to the prepared wells, raising the final fluid volume up to 240 µl which was the same level as the test wells.

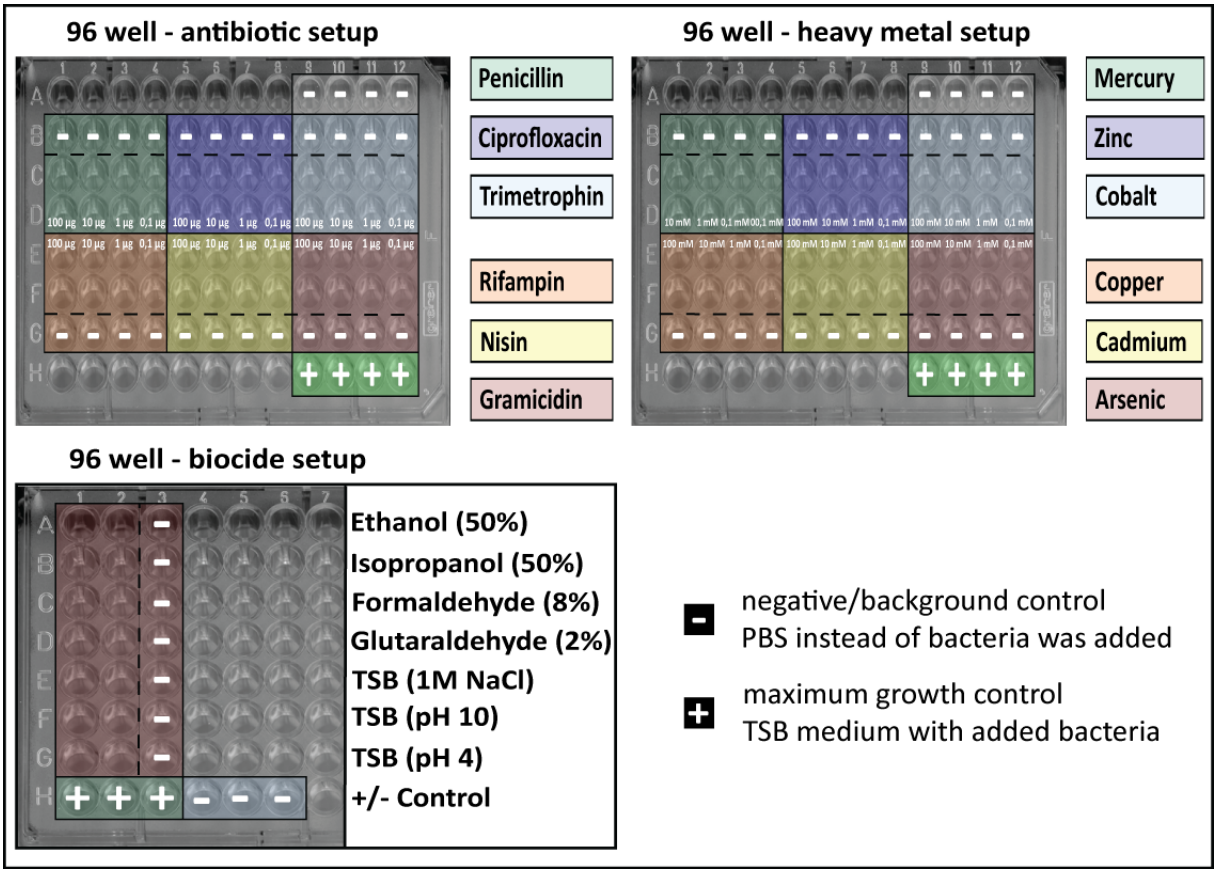

Figure 03: 96 well setup for the cultivable bacteria resistance tests
The Figure displays the substance and concentration schematic of the different resistance treatments (96 well). The background controls (no bacteria, only medium and additives) are marked by the white bars. The final absorption is calculated by averaging the absorption-values of the two inoculated wells (row 1-12, wells C & D respectively E & F) for each treatment and concentration.

2.4.4 H_2O_2 resistance testing

A liquid H_2O_2 protocol, developed by Riesenman & Nicholson (2000), was modified and used to examine the H_2O_2 resistance of the cultivable isolates.

833 µl of a washed and 1:10 in PBS diluted overnight culture was mixed with 167 µl of 33% H_2O_2 (to a final H_2O_2 concentration of 5%). The samples were incubated for 60 min at 37°C while shaking (Multiron 25, 200 rpm). The reaction was stopped by adding 900 µl of a bovine catalase solution (100 µg/ml) to 100 µl of the sample. The samples were appropriately serial diluted and plated out in duplicates on solid TSB medium. Growing bacteria (cfu) were counted after 20 h of incubation at 37°C. The surviving fraction was determined by the quotient of N/N_o, where N is the number of colony formers of the treated sample and N_o is the number of untreated cells forming colonies.

2.4.5 Desiccation resistance testing

Triplets of 40 µl of the undiluted and 10^{-2}, 10^{-4} and 10^{-6} diluted overnight cultures were pipetted into a 96 well plate and desiccated for seven days at 37°C.

After the desiccation phase 200 µl of fluid TSB media was added to each well and mixed thoroughly. The turbidity of the wells was measured after 20 h incubation at 37°C [see 2.4.2].

2.4.6 Heat tolerance resistance testing

500 µl of the washed, liquid overnight culture was transferred to a new Eppendorf tube, heat shocked (10 min, 80°C) and cooled down on ice. The bacteria were then appropriately diluted and plated out in petri dishes containing TSB agar. After 20 h of growth at 37°C the cfu of the samples were counted. The surviving fraction was determined by the quotient of N/N_o, where N is the number of colony formers of the treated sample and N_o is the number of untreated cells forming colonies.

2.4.7 UV-C resistance testing

Triplets of 40 µl of washed, 10^{-3} diluted overnight culture were dropped into a 96 well plate. This dilution was chosen to avoid artificial irradiation survival detection due to shadowing effects. The 96 well plates were exposed to UV-C radiation from a mercury low-pressure lamp (NN 8/15, Heraeus, Berlin, Germany) which peak emission line is at 253.7 nm [Figure 04]. Before and after the irradiation, the spectral intensity was measured using a UV-radiometer (UVX-Radiometer (UVX-Radiometer, UVP Ultra-Violet Products, Cambridge, UK)).

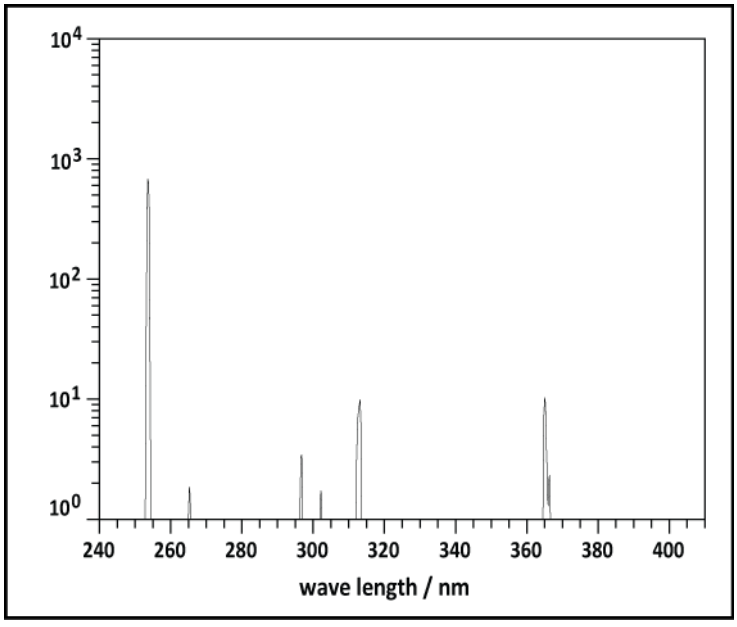

Figure 04: Emission spectrum of the mercury low-pressure lamp

By adjusting the distance between the UV-C lamp and the samples an effective flux of 180 J/m^2 was established and triplets of wells were irradiated with defined doses of 0 J/m^2, 100 J/m^2, 1000 J/m^2 and 2000 J/m^2. After the irradiation 200 µl of liquid TSB medium was added to each well. The 96 well plates were incubated for 20 h at 37°C and turbidity measured afterwards.

2.5 General molecular biology methods

2.5.1 Handling of equipment and solutions for work with nucleic acids

Thermostable solutions, glassware and other used utensils were autoclaved (121°C, 20 min) to inactivate any present nucleases. Surfaces which could not be autoclaved were either wiped down with 70% (v/v) ethanol or briefly flamed. Heat sensitive solutions were sterile filtered.

2.5.2 DNA concentration measurement

The optical density (OD) of solutions containing DNA was measured at 260 nm (OD_{260}) using a quartz-cuvette (GeneQuant^{Pro}, Amersham Biosciences, Freiburg, Germany). At this wavelength an OD_{260} of 1 is equal to a DNA-concentration of 50 µg/ml (Lottspeich and Zorbas, 1998). The purity of the DNA was calculated by the absorption-quotient established from two different wavelengths. For pure DNA-solutions the ratio for OD_{260} to OD_{280} should exceed 1.8 (Sambrook *et al.*, 1989). Lower values indicate a contamination of the DNA-solution with proteins.

2.5.3 Method for DNA-fragment characterization

2.5.3.1 Agarose gel electrophoresis

Agarose gel electrophoresis was used for the characterization of the amplified DNA after the PCR reaction. This technique allows the separation of DNA-fragments according to their size. The travel pace of the DNA fragments depends on several factors, i.e. the size of the DNA fragment, the used pore size of the gel (% agarose), the applied voltage and the salt concentration of the buffer. Depending on the size of the analyzed DNA molecules, gels containing 0.7% to 1.5% agarose were used. Agarose was dissolved in TAE buffer (45 mM Tris, 45 mM borate, 1 mM EDTA) by heating in a microwave for 2 min and poured into the gel chamber. After the gel was completely set (RT, 30 min) the samples were mixed with 1/6th of 6x loading buffer (Sigma gel loading solution (G2526-5 ml)) and loaded into the gel. Depending on the size of the DNA molecules the gel was run at 1-5 V/cm for 30 to 60 min. Once the DNA-fragments were sufficiently separated, the gels were incubated in an ethidium bromide bath (10 µg/ml) for 10 min and washed for another 10 min in a water bath. The DNA pattern was visualized by UV-light (Luminator (302 nm), UniEquip, Munich, Germany) and the results photo-documented.

2.5.4 Isolation of genomic DNA

2.5.4.1 Isolation of genomic DNA from swabs: efficiency testing setup

Four different concentrations (10^7, 10^4, 10^2, 10^1 cells/100 µl) of vegetative *Bacillus subtilis 168* overnight cultures were used during this part of the study. 100 µl of each suspension were either spotted on Alpha® swabs and incubated for an hour at RT before the DNA extraction or added directly to the lysis-buffer. The cell lysis was done either directly on the swabs, or on supernatants retrieved from the swabs by vortexing and sonification 35 kHZ (Qualilab (USR54H); Merck Eurolab, Germany) as per NASA standard assay, NPG: 5340.1D. The DNA of the lysed cells was then isolated as described in [5.2.2.4]. The efficiency of the DNA extraction was confirmed by 16S rDNA PCR and gel electrophoresis.

2.5.4.2 Isolation of genomic DNA using Invitrogens ChargeSwitch® Kit

After the cell lysis, 200 µl of ChargeSwitch® purification buffer were added and the sample mixed by gently pipetting up and down. Then 20 µl of ChargeSwitch® magnetic beads were added and the suspension once again mixed. The samples were incubated at RT for 5 min after which the DNA/bead compound was "immobilized" to the side of the Eppendorf tube using a magnet. The supernatant was removed and 500 µl of ChargeSwitch® wash puffer added. Another mixing step by pipetting followed and the magnetic beads were collected into a pellet using a magnet. Again the supernatant was discarded. The wash-step was repeated once more after which 50 µl of ChargeSwitch® elution puffer was added to the pellet. The samples were incubated at RT for 5 min and mixed a final time by pipetting. Using a magnet the beads were pelleted and the supernatant containing the DNA transferred to a clean Eppendorf tube. The DNA extract was stored at 4°C until further use.

2.5.4.3 Isolation of genomic DNA using Qiagen's QIAamp® DNA micro Kit

After cells were lysed 200 µl of 100% ethanol were added to the lysate and mixed by pulse-vortexing for 15 sec. The lysate was then transferred to the QIAamp MinElute column and centrifuge at 8000 rpm (miniSpin^{plus}, Eppendorf, Hamburg, Germany) for 1 min. The flowthrough was discarded and the QIAamp MinElute Column transferred to a clean 2 ml collection tube. 500 µl of buffer AW1 were added to the column which was afterwards centrifuged at 8000 rpm for 1 min. The samples were once more placed in a clean 2 ml collection tube and 500 µl buffer AW2 added. A centrifuge step at 8000 rpm for 1 min followed after which the columns were again transferred to a new clean tube. The samples were centrifuged at full speed 14,000 rpm for 3 min to dry the membrane completely and placed into a new tube.

30 µl of ddH_2O were carefully pipetted to the centre of the membrane and incubated at RT for 5 min. A final centrifuge step at full speed 14,000 rpm for 2 min collected the DNA from the column into the flow-through and which was stored at 4°C.

2.5.4.4 Isolation of genomic DNA using hot boil DNA extraction

One milliliter of liquid overnight culture was pelleted (500 rpm, 5 min) and resuspended in 200 µl of TE puffer containing 1% Tween-20. The samples were incubated at 95°C for 10 min and subsequently frozen at -60°C for 20 min. After this treatment, 200 µl of cold (4°C) Chloroform/Isoamylalcohol (1:24 (v/v)) was added and the phases mixed throughout by inverting the tubes. Phase separation was achieved by centrifuging for 5 min at 14,500 rpm (miniSpin^{plus}, Eppendorf, Hamburg, Germany).
The upper phase containing the DNA was transferred to a new Eppendorf tube and stored at 4°C.

2.5.5 DNA amplification

2.5.5.1 Amplification of DNA-fragments using Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR) was employed to create and amplify specific DNA fragments which were used for cloning and DNA sequencing. Hot-start *Taq* DNA Polymerase (Qiagen, Hilden, Germany) was used for the amplification. PCR reactions were prepared as instructed by the manufacturer (Qiagen, Hilden, Germany) and carried out in a 30 µl or 50 µl volume.

A 30 µl PCR reaction consisted of:

DNA	0.5-1 µg
oligonucleotides	100 pmol each
dNTP-mix	200 µM
Taq DNA polymerase	1 U
Reaction buffer (10x)	3 µl
H_2O_{dest}	filled to a final volume of 30 µl

The temperature cycles depended on the specific hybridization-temperature (x) and time needed for DNA polymerization (y). The following schematic illustrates the process:

1) hot-start	95°C	15 min
2) denaturing	95°C	1 min
3) annealing	x °C	1 min
4) DNA elongation	72°C	y min
35 cycles of step 2-4		
5) final DNA elongation	72°C	10 min

The bacterial universal primers 27F (5'- GAG TTT GAT C(AC)T GGC TCA G-3') (Lane, 1991) and 1492R (5'- GG (AT) TAC CTT GTT ACG ACT T -3') (Barns *et al.*, 1999; Blank *et al.*, 2002) were used to amplify a region of 1465 base pairs (bp) from the 16S rRNA gene. Using the following formula the optimal annealing temperature was calculated:

$T_{anneal} = 4(G + C) + 2(A + T)$ °C

T_{anneal} 27F = 58°C

T_{anneal} 1492R = 54°C

From the calculated optimal annealing temperatures of the two primers, 54°C was chosen as annealing temperature (x) for the amplification of the 16S rDNA gene fragment.

Due to the expected fragment length (1.5 kb) and the elongation speed of the *Taq*-polymerase (1 kb per minute), a two minute time interval was chosen as elongation time (y) for the 16S rDNA PCR. The PCR was carried out in a PTC-150 Minicylcer™ (MJ Research/Biorad, Munich, Germany).

The success of the PCR reaction was controlled by resolving the PCR products by agarose gel electrophoresis [7.3.1].

2.5.6 DNA Cloning

2.5.6.1 TA Cloning

16S PCR products were cloned using the the Qiagen PCR Cloning^{plus} kit. This system is designed for the direct cloning of PCR products into bacteria. The TA Cloning® vector, pDrive™, contains the lacZ-alpha complementation fragment for blue-white colour screening, ampicillin and kanamycin resistance genes for selection, and a versatile polylinker segment. The method relies on the non-template-dependent activity of *Taq* DNA polymerase, which adds a single deoxyadenosine (A) to the 3' ends of the PCR products. The linearized pCR™II vector contains 3' deoxythymidine (T) overhangs, which allows the PCR-product to ligate efficiently with the vector. Amplification of the fresh PCR-products was controlled by gel electrophoresis and the samples were purified using a QiaQuick gel cleaning Kit (Qiagen, Hilden, Germany). After purification and quantification, the ligation reaction was prepared. It consisted of the specific amount of PCR product (1-4 µl depending on DNA amount in the PCR reaction), 5 µl of 2x ligation buffer, 1 µl (50 ng) of pDrive™ vector, and was capped with sterile water to the total volume of 10 µl. The reaction was then incubated at 4°C for 2 hours. Afterwards 2 µl of the each reaction were added to a 50 µl aliquot of pre-thawed EZ competent *E. coli* cells (Qiagen, Hilden, Germany). The samples were gently stirred with a pipette tip and incubated on ice for 5 min. This was followed by a heat shock (42°C, 30 sec), after which the cells were placed, once more, on ice for 2 minutes. 250 µl of the SOC medium (RT) was added to the vial; and four times 50 µl aliquots were plated out on LB agar plates, containing 80 µg/ml X-gal, 50 µM IPTG and 35

µg/ml kanamycin. The transformed cells were incubated overnight at 37° and the plates checked for single white colonies the following day. The success of the transformations (white colonies) was furthermore controlled by 16S rDNA PCR amplification [2.5.5.1] and subsequent agarose gel electrophoresis [2.5.3.1].

2.5.7 DNA sequencing

2.5.7.1 16S rDNA sequencing of cultivable Isolates

Extracted genomic DNA [hot boil 2.5.4.4] from overnight cultures was used for 16S rDNA PCR amplification [2.5.5.1]. The generated Amplicons were checked by agarose gel electrophoresis [2.5.3.1] and purified using Qiagen's MinElute PCR purification kit (Qiagen, Hilden, Germany). The pure DNA solution was then sent and processed at AGOWA (Berlin, Germany). The amplicons were sequenced unidirectional using the primers 27F and 1492R; in effect, leading to two ca. 0.8-0.9 kb long DNA sequences which could be aligned and stitched together to obtain the 1.4 kb long DNA sequence of the 16S rDNA gene.

The obtained sequences were compared to existing sequences submitted to the public database (GenBank; http://www.ncbi.nlm.nih.gov/) to establish the taxonomic relation of the isolated organisms.

2.5.7.2 16S rDNA sequencing of uncultivable isolates (16S rDNA amplicons)

Following the successful amplification of the 1.5 kb long 16S rDNA fragment [2.5.5.1] from the original 18 Hydra facility samples, the amplicons were ligated into the pDrive (Qiagen, Hilden, Germany) vector using a TA-cloning approach [2.5.6.1]. After a 24 hour growth phase, 48 positive clones of each of the 18 selected sampling areas [Figure 01] were picked and grown in liquid LB media for 12 hours. Using a PCR reaction with the internal vector primers T7 and SP6, 12% of the picked clones were tested for successful integration of the 16S rDNA gene fragment. Transformation was only deemed successful and chosen for sequence analysis, if 80% or more of the tested clones exhibited the correct amplification product (1.5 kb). Of the successfully tested over night cultures, 96 well plates were created for sequencing. Therefore, 5 µl of each clone's liquid culture were added to 200 µl of TSB medium containing 10% glycerol and kanamycin (50 µg/ml). The clones were again incubated for 12 hours at 37°C without shaking and were then checked for growth. The 96 well plates were sealed with aluminum adhesive seals (ABgene Part #AB-0626) and stored at -80°C. Once all 96 well plates were created, the plates were shipped on dry ice at -30°C to Agencourt (Beverly MA, USA) and sequenced using Agencourt's Solid Phase Reversible Immobilization (SPRI) technology (DeAngelis *et al.*, 1995).

2.5.7.3 Processing of the obtained 16S rDNA sequences

After the forward and reverse DNA strands of the created 16S rDNA clones were sequenced [2.5.7] the data was processed further. First, the sequences were screened for unwanted vector information, which in turn was removed. Next, the two reads (forward and reverse) obtained from each clone were stitched together. This was possible for the reason that the average sequence read length was above 850 bp per strand. This creates an "overlap" (16S rDNA gene has a length of 1.5 kb) which can be used to stitch the two partial gene reads into one complete read. Therefore, the complementary sequence of the reverse read was created, aligned to the forward read and both reads stitched together by the present overlap. Each sequence was check for chimerical artefacts during the alignment process. This kind of artefacts can occur during the PCR amplification process [2.5.5.1] when DNA fragments from different organisms present in the environmental sample are combined into one sequence.

After the complete 16S rDNA sequence was established, the similarity of the sequence was compared to an in-house database (Caltech Novel Technology Report #4478) containing 5000 16S rDNA sequences from known bacterial strain types, as well as to the online NCBI/RDP II databases (http://www.ncbi.nlm.nih.gov/; http://rdp.cme.msu.edu).

The above-mentioned sequence processing was performed by the program STITCH (Caltech Novel Technology Report #4478). By applying this procedure to the obtained sequence, it was possible to identify the closest well-described bacterial species for each created 16S rDNA clone.

2.5.8 Construction of clone libraries

Three 16S rDNA clone libraries were constructed from the sequenced amplicons obtained from the direct DNA extraction of the samples collected at ESA's Hydra facility. Each clone library consists out of the pooled 16S rDNA sequences collected from one of the three different access-restriction zones established inside the Hydra facility. Library 1 encompasses amplicons from samples taken outside the restricted area (UC). Library 2 (CC) represents samples from inside clean room. Library 3 (CC+) consists out of 16S rDNA amplicons from samples collected inside the highly access-restricted ATV (automatic transfer vehicle) [3.2.2] which was positioned within the clean room.

2.5.8.1 Statistical analysis of 16S rDNA sequences clone libraries

2.5.8.1.1 Goods coverage and Chao's estimator

Three clone libraries, representing the different cleanliness levels inside the space hardware assembly facility (UC, CC and CC+), were created from the successfully sequenced clones and were subjected to statistical analysis. Therefore, Goods coverage (Good, 1953) and Chao's estimator (Chao, 1984) were calculated for each library. Operational taxonomic units (OTU's) were defined as clones exhibiting a 16S rDNA gene sequence similarity above 97% (Rosello-Mora and Amann, 2001). Goods coverage was calculated using the following equation:

$$C = \left[1 - \left(\frac{\#\text{singeltons}}{N}\right)\right] * 100$$

where C is the homologous coverage, singletons are the OTU's appearing only once in the specific library and N is the total number of examined clones.

The maximal theoretically occurring species within the three zones were calculated by applying Chao's estimator function:

$$S_{Obs} + \frac{F_1^2}{2(F_2+1)} - \frac{F_1 F_2}{2(F_2+1)^2}$$

S_{Obs} represents the overall number of identified OTU's and F_1 and F_2 correspond to the OTU's occurring respectively, only once or twice in the tested library.

The coverage percentage of identified species to theoretically present species (Chao's estimator) was calculated by:

$$(S_{coverage} = \frac{S_{Obs}}{S_{Chao1}})$$

2.5.8.1.2 Rarefaction curves

Rarefaction curves were used to compare the bacterial species diversity coverage between the three differently sized (N) clone libraries. To generate the rarefaction curves the number of observed OTU'S was plotted against the number of analyzed clones using the Analytic Rarefaction 1.3 software (http:www.uga.edu/~strata/software/index.html). OUT's were defined as mentioned in 7.7.4.1.

III. Results

International agreements (Outer space treaty, 1967) were developed and implemented to prevent the contamination of our solar bodies by mankind's exploratory projects. Today every space-faring nation has agreed to comply with these established policies. Depending on the mission profile and solar destination, strictly limited numbers of spores per space probe are allowed. These specific bioburden (spores per m^2) restrictions need to be met before a mission is allowed to lift off (COSPAR, 2002; NASA, 2005). In order to fulfil these regulations, the space agencies need to determine the microbiological profiles of their automated, as well as manned, spacecrafts on a continuous basis.
In recent years Europe, through ESA's exploration program, has started focussing on the inter-planetary exploration of our solar system. This endeavour will lead to a series of unmanned European explorative missions to Mars and other planets of our solar system. The frequency of such missions has also increased the need to carefully monitor the assembly process against unwanted contamination.

The goal of this work is the identification and characterization of a bacterial community found in a class 100k clean room. Therefore, existing clean room sampling procedures were critically examined, evaluated and improved to validate and lower the present detection limits for bacterial contamination. This is especially important in the low biomass environment at hand, where it is difficult to collect samples for a representative survey of the predominant bacterial community.
In the final part of this thesis, the resistance "behaviour" of the cultivatable fraction of the bacterial community was investigated whereby the effect this strict environment has on selection and/or adoption of the present organisms was investigated.

3.1 Analysis of NASA bioburden sampling procedure

Monitoring the bioburden of clean rooms is routinely done by swiping multiple $25cm^2$ areas of the facility. The samples are then incubated for a defined period of time and the total cfu counted. Depending on the respective mission profile, specific mandatory contamination limits need to be maintained and are checked constantly by swabbing. To understand the limitations of this kind of sampling technique and identify possible areas of improvement, the efficiency of the swabbing method was analyzed and verified.

3.1.1 Efficiency of swab sampling

Two standard treatments of detaching microorganisms from swabs were assayed for their efficiency. It was confirmed in advance that the sonication used for detaching would not alter the survival rate of the bacteria. 100 µl of a bacterial suspension containing a defined number of *Bacillus subtilis* spores was dripped onto the swabs which was followed by a 10 min incubation phase. As indicated in Figure 05, without any treatment most of the bacteria will stay attached to the swab. In the used set up, vortexing the swabs three times 10 seconds is slightly more effective than sonication of the swabs at 35 kHz for 2 min. Treating a swab with both methods only increases the amount of released bacteria slightly and no real synergistic effect was observed. Ultimately, the combination of both methods was deemed the most effective and used on all the samples from this point on.

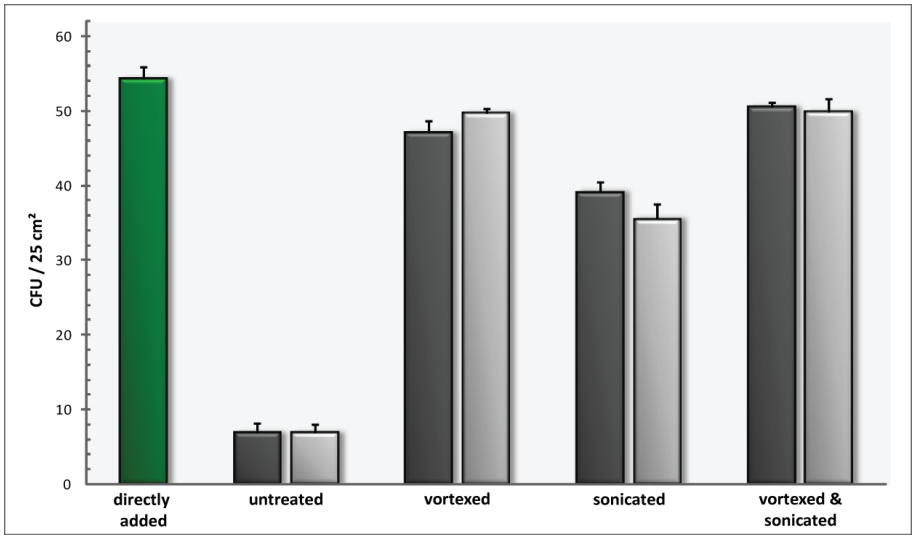

Figure 05: Effect of different treatments on the detaching efficiency from cotton (grey) & rayon (black) swabs

The swabs were spiked with approximately 10^2 spores +/- 12% (100 µl) and processed as follows: untreated for 10 min; vortexed 3 times 10 sec. during the 10 min incubation phase; sonicated for 2 min after 10 min incubation or vortexed (3 times 10 sec) during incubation and then sonicated for 2 min. The maximum amount of spores countable (green) was created by directly adding 100 µl of the spore suspension to the media. Each column represents the average of three repeats of 5 surface plates spiked, swabbed and counted. The bars indicate the simple standard deviation.

3.1.1.1 Effect of swab type on swabbing efficiency

The standard swab used for surfaces sampling consists of a head made out of cotton which is fixed to a wooden handle. Newer swabs integrate completely synthetic materials (rayon-head and plastic handle) to avoid possible contamination due to the use of natural products. The overall efficiency, consisting of the amount of bacteria taken up from the surface (retrieving) and the number of spores released from the swab after collection (detaching), of the rayon and cotton swabs was tested and compared. A 25 cm^2 area was spotted with 10^2 (+/- 12%) *Bacillus subtilis* spores and swabbed as described in [2.3.2.2.3]. A standard Petri dish (94/16 mm; Greiner bio-one, Austria) was used as

sampling surface. Comparing the retrieving efficiency of both swab types the rayon swab was able to take up 66% of the spotted bacteria whereas the cotton swab retrieved 57% of the microorganisms. The detaching rates, 61% for rayon and 69% for cotton, were very similar. Overall no major quality difference in the sampling efficiency between the two swab types was identified [Figure 06]. It was therefore concluded that the change from cotton to rayon swabs will not influence the sampling results.

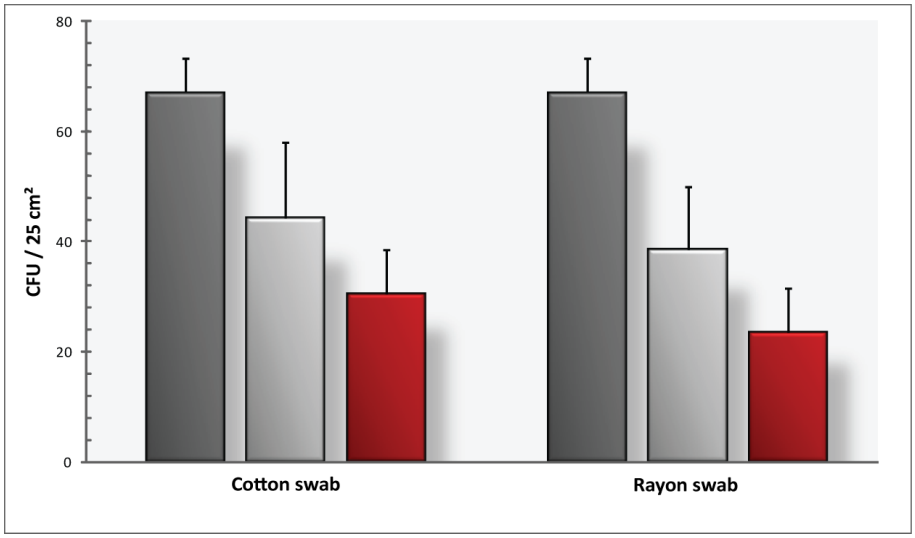

Figure 06: Comparison of swabbing efficiency between cotton and rayon swabs
The dark grey columns indicate the maximum amount of spores spotted. Dark grey columns represent the spores detached from the surface by swabbing. The red columns correspond to cfu counts after swab processing and subsequent incubation for 1 day at 37°C. Each column represents the average of three repetitions of five surface plates spiked, swabbed and counted. The bars indicate the simple standard deviation.

3.1.1.2 Effect of surface-materials on swabbing efficiency

Various materials are present in clean rooms during space probe assembly which offer bacteria a multitude of surface properties for attachment and colonisation. Therefore, the influence different surface materials have on the retrieving efficiencies of rayon swab was tested. Several space-certified materials, represented by Aluminium, V2A steel, MLI foil, Kapton and Teflon, were cut into 25cm^2 squares, spiked with *Bacillus subtilis* 168 spores and swabbed using the standard NASA procedure.

The results depicted in Figure 07 clearly indicate that the different materials used in this experiment had no pronounced effect on the overall sampling rate. Still the overall sampling efficiency was rather low. Only one half to one third of the bacteria present on the surface were actually retrieved by a swab. Again, from this number only 40% were released from the swab into the PBS solution and plated out. These two factors decrease the overall efficiency down to 30%.

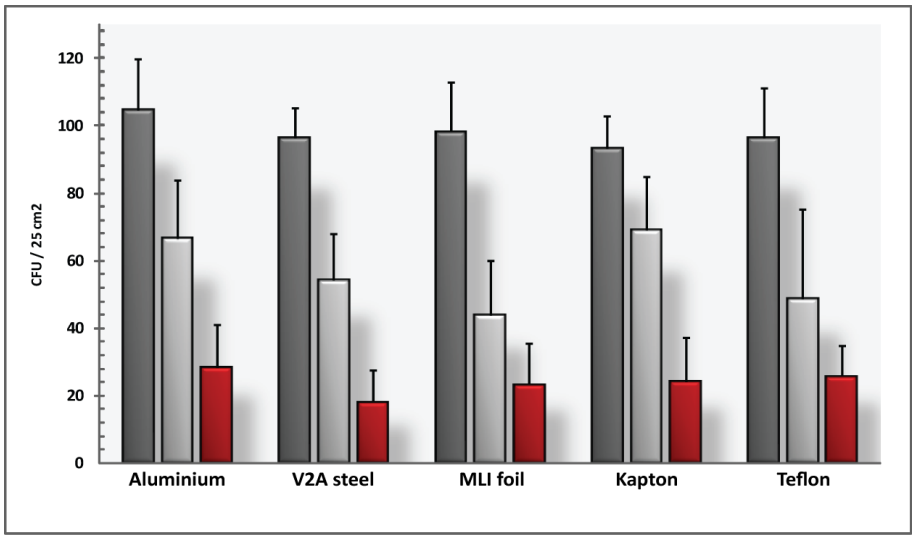

Figure 07: Swabbing efficiency of rayon swabs for different surface-materials
The dark grey columns indicate the maximum amount of spores spotted. Dark grey columns represent the spores detached from the surface by swabbing. The red columns correspond to cfu counts after swab processing and subsequent incubation for 1 day at 37°C. Each column represents the average of three repetitions of five surface plates spiked, swabbed and counted. The bars indicate the simple standard deviation.

3.1.2 DNA extraction and isolation from swab samples

After the swabbing efficiency and sample processing for the cultivable bacteria community was established, effective and sensitive protocols for the molecular processing of the swabs needed to be developed. For this purpose, several cell lysis and DNA extraction protocols were tested and assembled into a protocol delivering a low and well defined DNA detection limit.

3.1.2.1 Establishment of a cell lysis protocol

Extracting DNA from environmental sites is especially challenging. Most samples consist of a wide variety of organisms, combining a mix of fragile and sturdy organisms. A cell lysis protocol needed to be developed to open robust gram-positive bacterial cells and resilient dormant forms like spores. In the end, a combination of treatments including chemical (edetic acid and sodium dodecyl sulphate, pH 8.3), enzymatic (Lysozyme) and physical (freeze/thaw cycles) cell lysis steps were chosen as standard procedure for direct cell lysis from swabs [2.3.2.2.4].

After the cells were lysed, the released DNA was precipitated and washed as described in 2.3.2.2.4. Successful DNA extraction was confirmed by agarose gel electrophoresis [2.5.3.1]. Using this protocol, it was possible to extract PCR quality DNA from down to 10^2 spores [Figure 08].

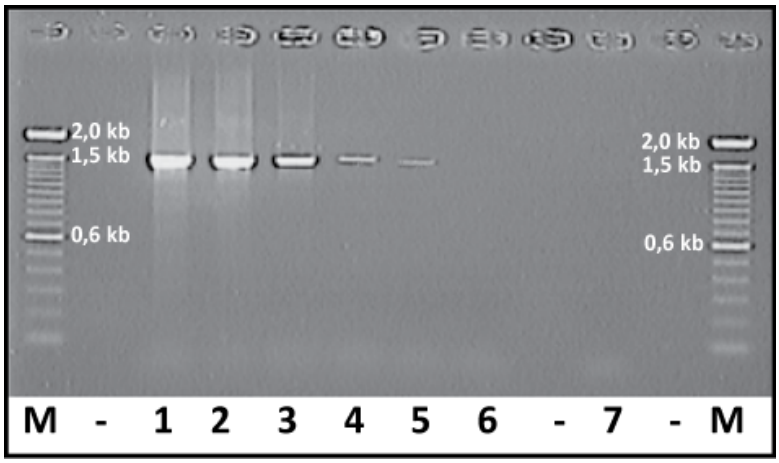

Figure 08: Extraction limit of established cell lysis protocol
1.5% agarose gel of 16S rDNA PCR product from the supernatant of a lysed dilution series of *Bacillus subtilis* spores.
Lane M: marker; lane 1: 10^6 spores; lane 2: 10^5 spores; lane 3: 10^4 spores; lane 4: 10^3 spores; lane 5: 10^2 spores; lane 6: 10^1 spores; lane 7: blank sample (lysis extraction negative control).

3.1.2.2 Comparison of three DNA extraction methods

After the cell lysis protocol was established, DNA extraction methods were tested for their DNA recovery efficiency. To test these methods four different concentrations (10^7, 10^4, 10^2, 10^1 cfu/100 µl) of vegetative *Bacillus subtilis* 168 cells were lysed as described in 2.3.2.2.4. The bacterial DNA from these samples was then extracted using three different techniques. Two of these methods are commercially available kits (Qiagen QIAamp DNA Micro Kit and Invitrogens ChargeSwitch® Kit). While Qiagen QIAamp DNA Micro Kit extracts and purifies the DNA using a column based approach, Invitrogens ChargeSwitch® uses charged magnetic beads to bind selectively to the DNA. These two kits were tested for their extraction capabilities and compared to a third extraction method (hot boil) which is a well-known fast and reliable procedure. DNA recovery protocols were conducted as described in 2.5.4, and the efficiency of the DNA extractions was confirmed by PCR amplification. 3 µl of the respective DNA extractions were used as template in a 16S PCR-reaction [2.5.5.1], and the amplification reaction visualized by agarose gel electrophoresis.

The hot boil DNA extraction method was able to obtain PCR grade DNA from samples containing 10^4 or more bacteria. Below this concentration no positive amplification was evident. In contrast, both kits were able to reliable produced PCR grade DNA from a cell concentration as low as 10^2 bacteria. Comparing the overall amplification band strength of all three methods showed that, under the conditions used, the QIAamp DNA Micro Kit is the most effective DNA extraction method, leading to the strongest amplification product (DNA band at 1.5 kb) at each dilution. Furthermore when comparing the handling of both kits, the results generated with the QIAamp DNA Micro Kit were more consistent and reliable. The above-mentioned results led to the selection of this kit for DNA extraction from this point on.

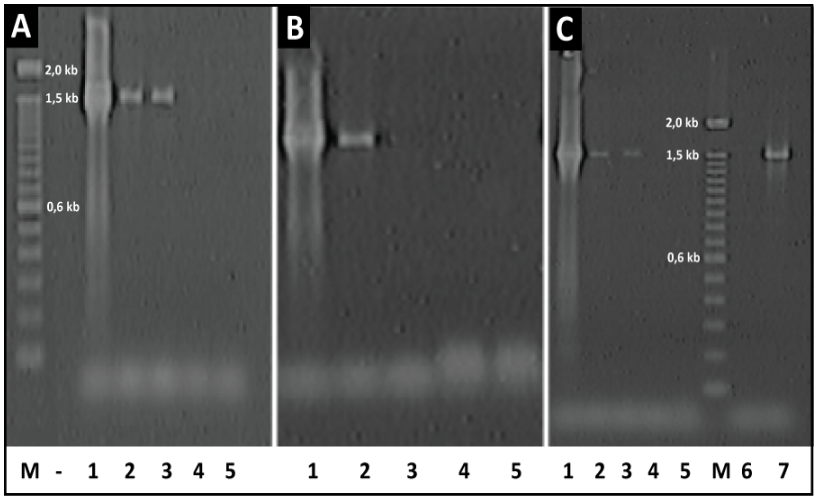

Figure 09: Comparison of the three tested DNA extraction methods
Agarose gels (1.5%) of 16S rDNA PCR product using the primer 27F and 1492R. Three different DNA extraction methods were used for DNA extraction. A: QIAamp DNA Micro Kit (Qiagen); B: ChargeSwitch® Kit (Invitrogen); C: hot boil method. Lane 1: 10^7 cells; lane 2: 10^4 cells; lane 3: 10^2 cells; lane 4: 10^1 cells; lane 5: blank PCR control; lane 6: 2nd blank PCR control; lane 7: positive PCR control; lane M: marker.

3.2 Sampling of a European class 100k clean room

To accurately survey the bacterial community present in a clean room during space hardware assembly, a facility had to be located where sampling could take place during active use of the clean room. A suitable facility was found at ESTEC, Noordwijk the Netherlands, where ESA operates a class 100k clean room: the Hydra facility. The facility was sampled in May/June of 2005 while it was being used to assemble and test the automated transfer vehicle (ATV). This hardware will be used to supply the international space station (ISS) with expandable goods and other necessary materials.

3.2.1 Sample collection at ESA's Hydra facility

Two sampling trips (31.05.2005 and 14.06.2005) were carried out to collect representative samples of the existing bacterial community of the space hardware test facility and to establish the present bioburden.

18 surface areas, chosen from the different cleanliness levels within the facility, as well as the air inside and outside the restricted area, were surveyed during this study to compile an overview of the bacterial contamination present in this ESA facility. The exact location of the different sample sites is depicted in Figure 01 [2.3.3.1]. More details on the sampling locations can also be found on this thesis' Data-CD.

Overall, 80 swabs, as well as 12 air samples were collected for analysis and provided the basis from which the class 100k clean room bacterial community was established.

3.2.1.1 Cultivation of aerobic heterogeneous bacteria

Bacteria from the clean room were collected and isolated to identify the fraction of the community which could grow under standard laboratory conditions. Bacteria were cultivated aerobically on R2A agar plates and incubated at 25°C for seven days. The cfu count of the heterogeneous bacterial community was noted on day two and seven of the incubation phase. The actual number of bacteria grown for each sampled site can be found in Table 08 for the first sampling. Table 09 depicts the cfu's detected during the second sampling.

Overall, it was possible to grow viable bacteria from each of the different sample sites tested during this study. After the incubation phase, some of the sample plates exhibited a bacterial count of 200+ cfu's and were marked as "too numerous to count" (TNTC).

Table 08: Cfu of cultivable aerobic heterotrophic and heat tolerant bacteria at the first sampling of ESA's Hydra facility (31.05.05)

	heterotrophic cfu count after incubation for 7 days at 25°C				**heat tolerant cfu count** after incubation for 7 days at 32°C			
Sample	Plate 1	Plate 2	Average	STDEV	Plate 1	Plate 2	Average	STDEV
01	3	0	1.50	2.12	-	-	-	-
02	9	6	7.50	2.12	1	0	0.50	0.71
03	5	9	7	2.83	1	1	1.00	0.00
04	2	0	1	1.41	3	0	1.50	2.12
05	30	23	26.50	4.95	9	7	8.00	1.41
06	TNTC	TNTC	TNTC	-	1	2	1.50	0.71
07	1	0	0.50	0.71	-	-	-	-
08	TNTC	TNTC	TNTC	-	1	1	1.00	0.00
09	1	0	0.50	0.71	-	-	-	-
10	1	1	1	0.00	-	-	-	-
11	TNTC	TNTC	TNTC	-	2	0	1.00	1.41
12	1	6	3.50	3.54	-	-	-	-
13	0	0	-	-	-	-	-	-
14	1	0	0.50	0.50	2	2	2.00	0.00
15	1	1	1	0.00	-	-	-	-
16	1	0	0.50	0.71	-	-	-	-
17	2	1	1.5	0.71	-	-	-	-
18	1	40	20.50	27.58	-	-	-	-
19	0	0	-	-	-	-	-	-
20	2	0	1	1.41	-	-	-	-

3.2.1.2 Cultivation of aerobic heat tolerant and spore forming bacteria

To select and grow spore forming or heat tolerant organisms the samples were heat shocked (80°C, 10 min) before plating out on TSB agar [2.3.4.3]. The samples were then aerobically cultivated for seven days at 32°C and the cfu's counted at day two and day seven. The actual cfu count for the sampled sites is presented in Table 08 for the first sampling and Table 09 for the second sampling trip.

It was possible to grow viable, heat tolerant bacteria from most of the Hydra facility's samples sites but, as expected, the overall cell count of heat tolerant bacteria is about a factor 10 lower than the cell count for the untreated bacterial population. Since the average cell count of heat tolerant bacteria was below one colony per sample site, no meaningful comparison between the controlled and uncontrolled area could be conducted.

Table 09: Cfu of cultivable aerobic heterotrophic and heat tolerant bacteria at the first sampling of ESA's Hydra facility (14.06.05)

	heterotrophic cfu count after incubation for 7 days at 25°C				**heat tolerant cfu count** after incubation for 7 days at 32°C			
Sample	Plate 1	Plate 2	Average	STDEV	Plate 1	Plate 2	Average	STDEV
01	7	6	6.5	0.71	4	3	3.50	0.71
02	1	1	1	0	1	0	0.50	0.71
03	0	0	-	-	0	0	-	-
04	0	0	-	-	0	0	-	-
05	10	3	6.5	4.95	5	1	3.00	2.83
06	0	0	-	-	0	0	-	-
07	0	0	-	-	0	0	-	-
08	2	0	1	1.4	1	0	0.50	0.00
09	0	0	-	-	0	0	-	-
10	0	0	-	-	0	0	-	-
11	0	0	-	-	0	0	-	-
12	2	3	2.5	0.71	0	0	-	-
13	0	0	-	-	0	0	-	-
14	0	0	-	-	0	0	-	-
15	1	0	0.5	0.71	0	0	-	-
16	2	0	1	1.4	0	0	-	-
17	0	0	-	-	0	0	-	-
18	5	0	2.5	3.54	1	0	0.50	0.71
19	0	0	-	-	0	0	-	-
20	0	0	-	-	0	0	-	-

3.2.1.3 Cultivation of aerobic heterogeneous air bacteria

The air of the class 100k clean room facility was sampled using an Airport MD8 (Satorius). The actual sample sites outside (Air 1 & Air 2) and inside (Air 3 & Air 4) the controlled area are marked down in Figure 01 [2.3.3.1]. To collect as many microorganisms as possible from the air samples, the filters were not tested for heat tolerant organisms and only processed aerobically with their exposed face down on the R2A agar plates (25°C for seven days). The actual cfu count of the air samples is depicted in Table 10. It was possible to cultivate bacterial strains from all the taken air samples. The number of observed cfu's decreased considerably from samples taken outside the clean room to those collected within the restricted area.

Table 10: Average cfu of cultivable aerobic heterotrophic air bacteria from both sampling trips to ESA's Hydra facility (31.05.05 and 14.06.05)

Sample	Area	Sampling 31.05.05	Sampling 14.06.05
Air 1, 2	Outside the clean room, near the exit to the hallway	68	23
Air 3	Inside the clean room, near the exit to the changing room	1	5
Air 4	Inside the clean room, near the exit to the second clean room	6	1

3.2.2 Contamination level & bioburden determination of ESA's Hydra facility

To determine the bacterial contamination level inside the Hydra facility the samples were processed as described in 2.3.4 and, either heat shocked (10 min 80°C) or directly plated out. Air samples were only cultivated directly without the application of a heat shock. The cfu grown after seven days are depicted in Figure 10 for the first sampling and in Figure 11 for the second sampling.

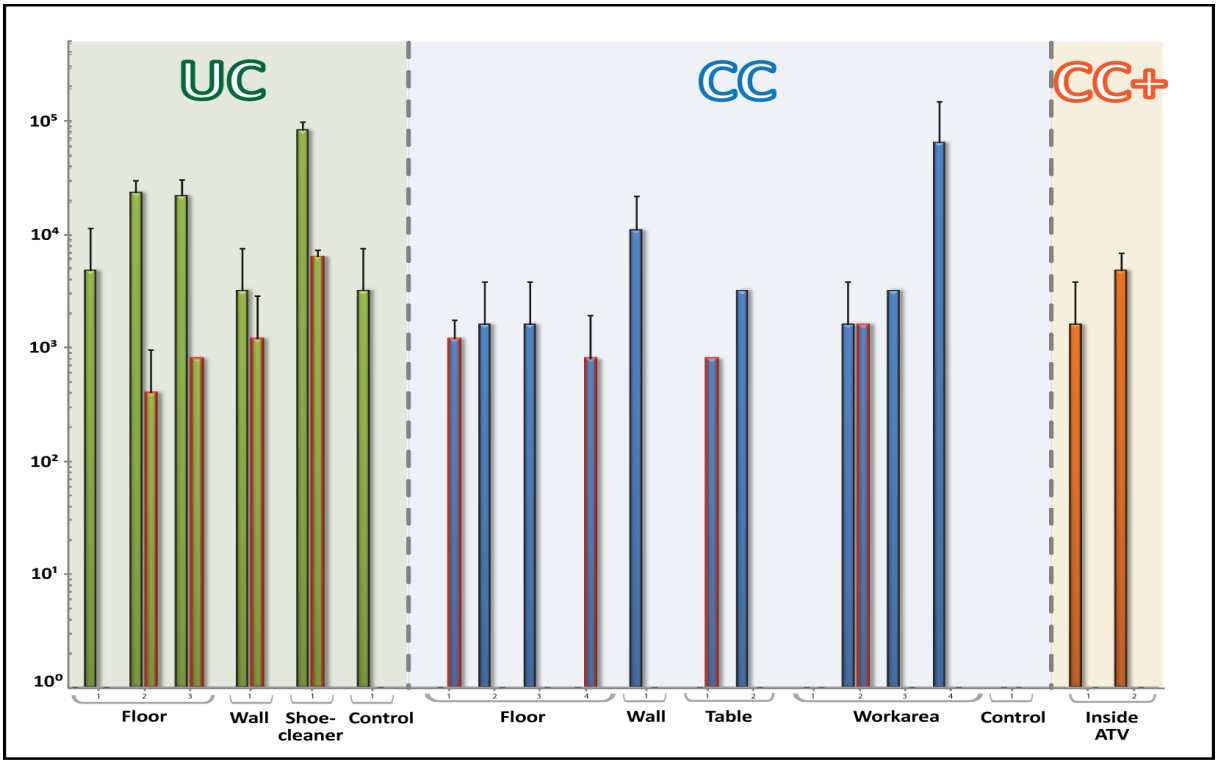

Figure 10: Bioburden & contamination level of ESA's Hydra facility (first sampling, 31.05.05)
Number of heterotrophic and heat tolerant cfu's per square meter. The samples were grouped into three zones representing the different cleanliness levels present inside ESA's Hydra facility: outside the clean room (UC = green), inside the clean room (CC = blue) and inside the highly access-restricted ATV (CC+ = orange). Cfu of heat tolerant bacteria (bioburden) are represented by red bordered columns.

The contamination level (cfu of heterogeneous bacteria/m^2) and bioburden (cfu of heat tolerant bacteria/m^2) (Nasa NPG: 5340.1D) of the Hydra facility was then extrapolated from the identified bacterial colonies. The samples were, furthermore, divided into three distinct categories depending on the access-restriction requirements in effect at the tested areas. The area (UC) outside the class 100k clean room represents the uncontrolled environment inside the Hydra facility. This category is derived from 16S rDNA sequences of the samples Hydra 1-5. The next group (CC) consists out of 16S rDNA sequences from the samples Hydra 6-15 and Hydra 18 taken from inside the clean room. The category (CC+) was created by pooling the samples taken from inside the ATV (Hydra 16 & Hydra 17). Access of the ATV was restricted to one person at a time wearing a full body suit. These protocols represent the strictest admission level applied at the Hydra facility.

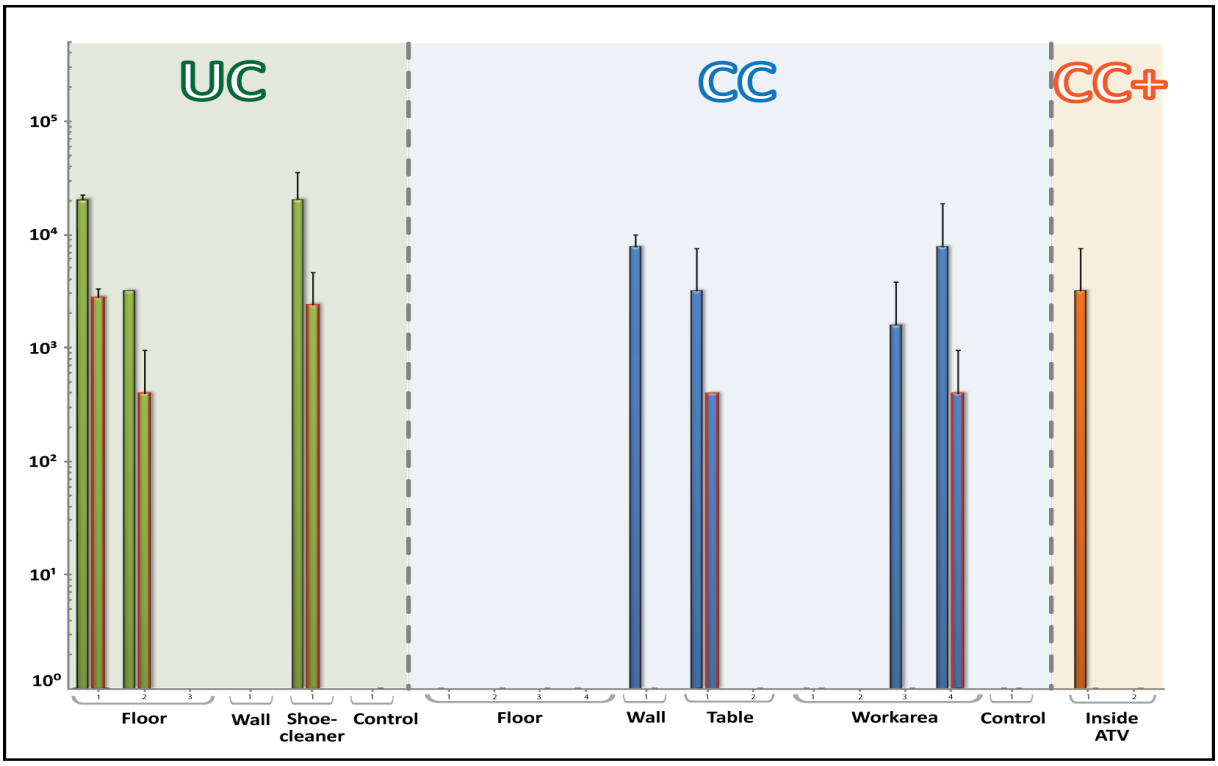

Figure 11: Bioburden & contamination level of ESA's Hydra facility (second sampling, 14.06.05)
Number of heterotrophic and heat tolerant cfu's per square meter. The samples were grouped into three zones representing the different cleanliness levels present inside ESA's Hydra facility: outside the clean room (UC = green), inside the clean room (CC = blue) and inside the highly access-restricted ATV (CC+ = orange). Cfu of heat tolerant bacteria (bioburden) are represented by red bordered columns.

The average contamination level inside the clean room of 10^{3-4} bacteria per m^2 as well as the bioburden of 10^{2-3} (heat tolerant bacteria) per m^2 lay within the documented bacterial contamination of industrial used class 100k clean rooms (Favero *et al.*, 1968; Puelo *et al.*, 1973).

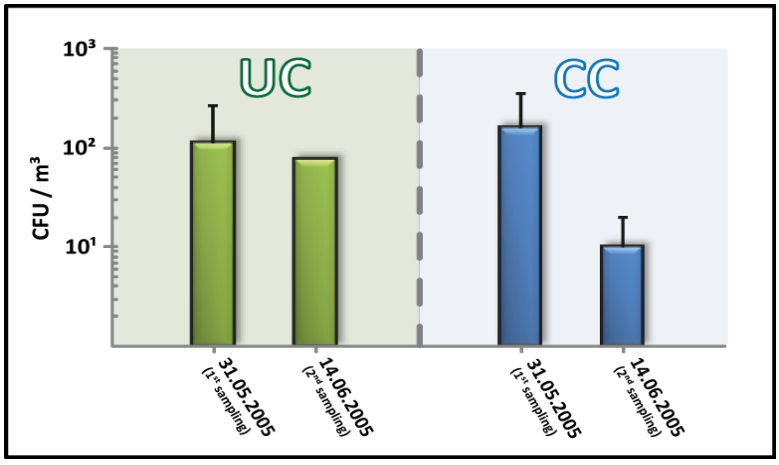

Figure 12: Contamination level of air samples collected at ESA's Hydra facility
Number of cultivated heterotrophic air bacteria (cfu) per cubic meter. The samples were taken outside (UC = green) or inside (CC = blue) the clean room.

3.2.3 Obtaining pure cultures and compilation of an environmental isolate-archive

After incubating each sample for seven days the plates were inspected for grown bacteria exhibiting different morphologies. Representatives of each of the different morphologies were picked from each sample and streaked out again onto separate agar plate. This process was repeated until each plate exhibited only colonies of a single morphology. Altogether 82 bacteria could be isolated from the Hydra facility. As expected the largest number of bacteria were isolated from the untreated surface samples (64%), whereas 24% of the isolates could be isolated from the heat shocked part of the surface samples. Only 12% of the isolates were acquired from the air samples.

Once pure cultures were established, cryostocks were created from each isolate and stored at -70°C until further use [2.3.1.3].

3.3 Analysis of the Hydra facility's cultivable bacterial fraction

An overnight culture was prepared from each bacterial strain's cryostock. 1.5 ml of the suspension was processed and the DNA extracted the next day as described in 2.5.4.4. The degenerated primer 27F & 1492R were used for amplification of the variable region (V3) of the *Bacteria* domain specific 16S rDNA gene (Neefs *et al.*, 1990). After the gene amplification was confirmed, the DNA was purified (Qiagens MinElute® kit) and sequenced (AGOWA, Berlin, Germany).

Applying the above mentioned strategy, PCR grade DNA was extracted from the collected bacterial strains. Furthermore, it was possible to associate all of the 82 collected bacteria from ESA's Hydra facility to known bacterial type strains with a certainty of ≥97%. Thus, the collected cultivable bacterial fraction could be identified as detailed as the species level.

3.3.1 Analysis of phylogenetic composition of collected cultivable bacteria

From the DNA sequences of the 82 isolates, 18 different bacteria species present in Hydra facility's clean room could be identified [Table 11]. The species can be associated with three phyla. The majority of the cultivated bacteria (78% of the species) belong to the *Firmicutes* phylum encompassing 14 of the 18 identified bacterial species. The organisms in this phylum are affiliated with three bacterial families: the *Staphylococcaceae* (28%), the *Bacillaceae* (39%) and the *Paenibacillaceae* (11%). The *Actinobacter*, the second identified phylum, constitutes 11% of the cultivable species. The collected strains belong to the family of *Corynebacterineae* (5.5%) and *Micrococcineae* (5.5%). The third discovered phylum, the *Proteobacteria*, represents the final 11% of the cultivable bacterial species and is comprised of the *Pseudomonadaceae* (5.5%) and *Methylobacteriaceae* (5.5%) families.

While representatives of the *Actinobacter* and *Firmicutes* could be detected inside, as well as outside the restricted area; *Proteobacteria* strains were only collected from outside the class 100k clean room. Otherwise, no profound shift in species ratio between the area inside and outside the clean room was observable. More details about the identified cultivable bacterial species can be found in Table 20 & 22 of the appendix, or on the Data-CD.

Table 11: Phylogenetic composition of cultivable bacteria collected at ESA's Hydra space craft assembly & testing facility

Phylum/class	**Number of clones**	**% overall**
Firmicutes	**14**	**78.00%**
Bacilli	14	78.00%
Proteobacteria	**02**	**11.00%**
Alpha-Proteobacteria	01	5.50%
Gamma-Proteobacteria	01	5.50%
Actinobacteria	**02**	**11.00%**
Actinobacteria (class)	02	11.00%
Sum	**18**	**100%**

3.3.2 Morphology and 16S rDNA comparison between bacterial strains of one species

From the samples taken at the Hydra facility it was possible to cultivate and isolate over 80 bacteria. The isolates were classified by their morphology as well as their 16S rDNA sequence. Once the isolates were identified, the different strains of the same species were compared among each other to check for noticeable individual changes in phenotype, 16S rDNA or resistance behaviour [3.6].

Figure 13: Phenotype comparison of individual strains identified as *Staphylococcus epidermidis*
Picture of isolated individual *Staphylococcus epidermidis* strains collected from each of the three different cleanliness zones established inside ESA's Hydra space craft assembly and test facility. The colors represent: green = outside the clean room (UC), blue = inside the clean room (CC) and orange = inside the highly access-restricted controlled ATV (CC+).

Single individual clones of the same bacterial strain isolated from inside, as well as outside the clean room exhibited no substantial variations in their morphology (Figure 13 displays an example of this observation). When the clones were compared on the genetic level (16S rDNA, example Figure 14), again minor inconsistencies between individual clones were detected. These variations were well within the error of the sequencing method or of natural variation and should not be considered major differences in genotype between the different strains of one species.

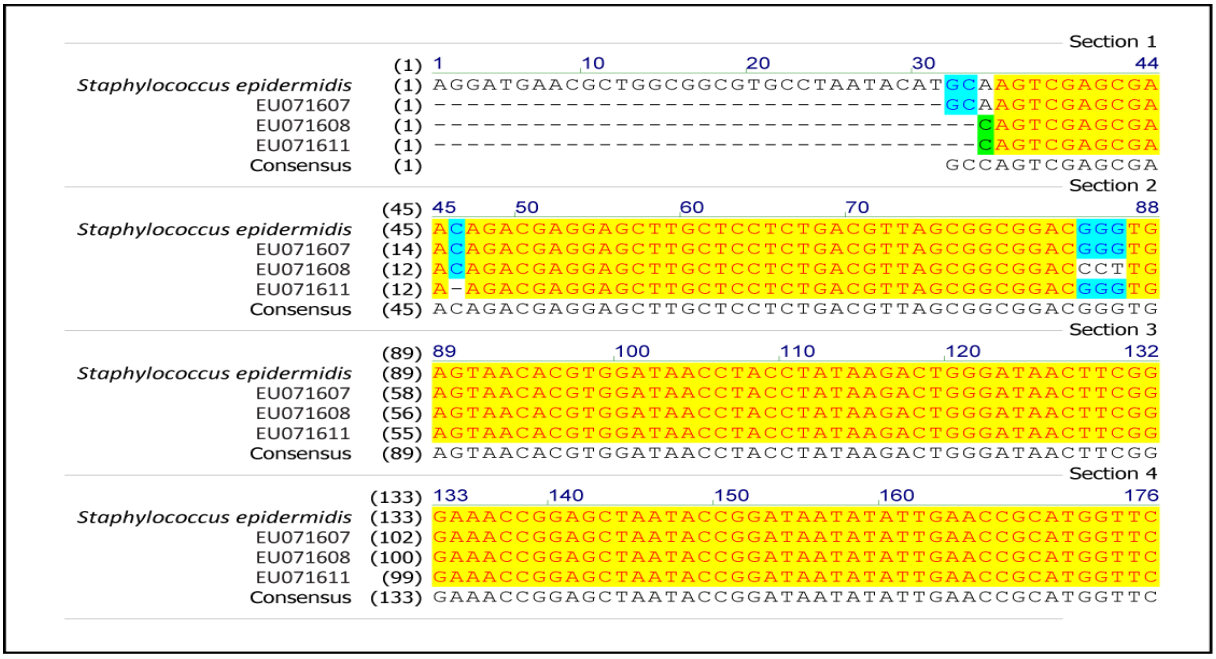

Figure 14: Genotype comparison of individual clones of *Staphylococcus epidermidis*
Extract of the 16S rDNA alignment of three *Staphylococcus epidermidis* strains collected from the different cleanliness level (UC, CC and CC+) within ESA's Hydra assembly and test facility.

3.4 Analysis of the Hydra facility's uncultivable bacterial fraction

3.4.1 Direct DNA extraction from the Hydra facility samples

When testing the contamination of a clean room used for space probe assembly the established standard techniques are primarily based on a cultivation-based approach. Nowadays, it is known that only a small percentage of the actual existing organisms can be cultured under standard laboratory conditions leaving the majority of present microorganisms undetected. Therefore, modern techniques were incorporated into the sampling procedure, as described in 2.3.2.2.4, to identify organisms by their biomarkers on the molecular level. This approach provides the opportunity to detect and identify most of the present microorganism without the need for a cultivation step. Overall, the integration of different classes of contamination detection techniques into the survey brings the process a step closer to the ultimate goal of establishing a realistic contamination assessment for the tested clean room.

3.4.2 Cloning of environmental bacterial 16S rDNA genes

3.4.2.1 Isolation of genomic DNA from the Hydra facility samples

Once the samples were processed for cultivable bacteria, the remaining supernatant (approx. 300 µl) and swab heads were treated as described in 2.3.2.2.4 to extract existing genomic DNA. During sample preparation special attention was paid to prevent and control the samples against contamination from the ubiquitously present environmental 16S rDNA. In the course of DNA extraction and amplification, two separate negative controls were processed alongside the samples to identify any possible contamination. Using the newly established protocols, it was possible to extract PCR grade DNA from all 18 different Hydra facility sample sites.

3.4.2.2 Amplification of environmental 16S rDNA genes

After the DNA was prepared, a PCR reaction [2.5.5.1] using the universal bacterial primers 27F & 1492R was carried out to amplify the existing 16S rDNA genes from the environmental samples. All PCR reactions from the extracted genomic DNA showed, exclusively DNA fragments of the expected size of 1.5 kb, while the respective negative controls did not exhibit any amplification product. Figure 15 represents an example of a successful DNA extraction and subsequent 16S rDNA amplification which was visualised by agarose gel electrophoresis.

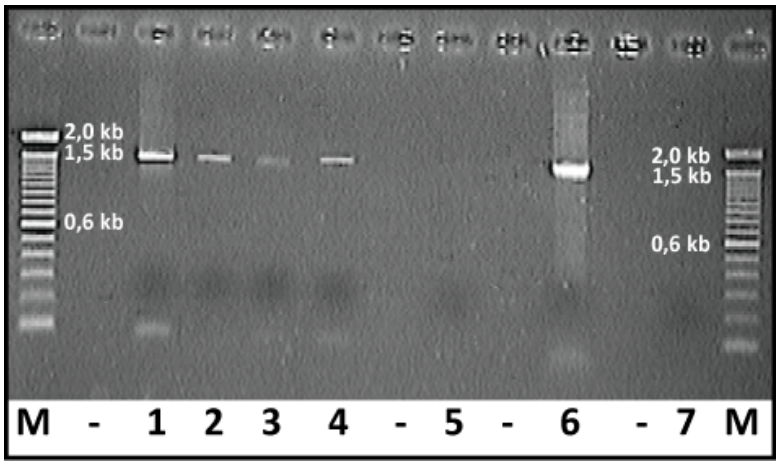

Figure 15: Direct DNA extraction from Hydra facility samples
1.5% agarose gel of 16S rDNA PCR product using primer 27F and 1492R from different Hydra facility samples sites (ss).
Lane M: marker; lane 1: ss Hydra facility 1; lane 2: ss Hydra facility 2; lane 3: ss Hydra facility 11; lane 4: ss Hydra facility 12; lane 5: blank DNA extraction control; lane 6: positive PCR control (genomic DNA from *B. subtilis*); lane 7: second blank DNA extraction control.

3.4.2.3 Sequencing quality and efficiency analysis

After the DNA was successfully isolated from the collected Hydra facility samples [3.4.2.2], the variable V3-Region of the bacterial 16S ribosomal gene (Neefs *et al.*, 1990) was amplified by PCR using the primers 27F and 1492R [Table 02]. The success of the amplification was controlled by agarose gel electrophoresis to screen for artificial PCR-products, like high genomic DNA background

or chimerical DNA sequences which can originate from merged rDNA sequences of two organisms. As the applied cell lysis protocols were specifically assembled to prevent a strong fragmentation of the DNA, which is known to increase the amplification of chimeric sequences (Liesack *et al.*, 1991), only a minor fraction of the PCR reactions exhibited such artefacts [Table 12].

3.4.2.4 16S rDNA amplicon quality analysis

Forty-eight successfully transformed clones were picked from each sampled location for sequence analysis. Altogether 864 clones were processed at Agencourt (Beverly MA, USA). Nucleotide sequencing of the inserts was carried out on both strands (forward and reverse) so that the cloned 1.5kb long 16S rDNA sequence could be stitched together from the two single reads. Table 12 provides a quality overview of the generated amplicons. The sequencing was successful in over 92% of the cases, generating DNA sequence from 808 of the clones.

In 90% of the analysed clones high quality sequences, consisting of ≥ 750 identifiable bp, were obtained. From 611 of these clones full length amplicons, spanning the whole 16S rDNA inserts, could be stitched. The amplified 16S rDNA region contains V3 variable region allowing an effective sequence comparison against existing 16S rDNA databases. Using a dataset (Caltech Novel Technology Report #4478) containing more than five thousand bacterial type strains 16S rDNA sequences, 415 of the clones could be associated to known bacterial species with a sequences similarity of over 97%. The full 16S rDNA identification details and obtained 16S rDNA sequences can be found on the Data-CD.

Table 12: Sequencing analysis of the cloned bacterial environmental 16S rDNA sequences

Direct 16S rDNA cloning	Numbers	% overall
Sequence quality	**1616**	**100%**
high quality sequences (<750 bp)	1459	90.28%
low quality sequences (>750 bp)	157	9.72%
Clones created from sequences	**808**	**100%**
Successfully created clones by stitching R & F Sequences	611	75.6%
Successfully stitched, but below 1k bases compared	131	16.2%
Could not create clones by stitching R & F Sequences	66	8.2%
Sequence similarity of clones to offline 16s rDNA database	**611**	**100%**
Above 97%	415	67.92%
Below 97%	196	32.08%

3.4.3 Construction of environmental 16S rDNA clone libraries

Once the successfully created amplicons were sequenced and quality controlled they were utilized to create distinct 16S rDNA clone libraries corresponding to the cleanliness level present in ESA's Hydra facility. Three clone libraries were established from the 611 fully sequenced 16S rDNA clonal inserts [2.5.6]. The sequences were divided into two different classes depending on the sequence similarity to known bacterial species. Clones exhibiting a similarity below 97% were not used in this study for species analysis as they could not be sufficiently identified. The second group, featuring a sequence similarity above 97% to known bacterial typestrains, were considered fully-identified operational taxonomic units (OTU), representing a bacterial species.

Within these three libraries 80 different OTU's were identified and used for further phylogenetic analysis.

3.4.3.1 16S rDNA clone library quality analysis

The created clone libraries were subjected to several analytical tests to estimate the extent to which these libraries cover the actual phylogenetic diversity present in the clean room. First the sample coverage of the three created clone libraries was calculated according to Good (Good, 1953). The three libraries (UC, CC and CC+) covered the majority of the estimated species diversity inside the collected samples, with C_{Good} = 89%, 95% and 92% respectively [Table 13].

The identified OTU's were further used to compile a rarefaction analysis (Heck *et al.*, 1975) of the three libraries to approximate the relative abundance of phylotypes that would be expected per level of effort [Figure 16]. The rarefaction curves of the clone libraries assembled from inside the clean room (CC and CC+) possess a levelling character, indicating that the present bacterial diversity is nearly (CC) or almost (CC+) completely represented by these libraries. The rarefaction curve computed from the UC clone library, in contrast, featured an unpronounced curvilinear plot indicating that the species diversity of the uncontrolled environment was incompletely sampled.

Chao's estimator (Chao, 1984) was used as a non-parametric estimator to provide an unbiased approximation of the total present bacterial diversity. The estimated maximal number of species by Chao's abundance-based coverage estimator was 60, 69 and 22 phylotypes for the UC, CC and CC+ library, respectively. The following formula was used to analyze the percentage of identified species:

$$\left(S_{coverage} = \frac{S_{Obs}}{S_{Chao1}} \right)$$

It is evident that the libraries created from inside the restricted area provide, yet again, a more complete representation of the present bacterial community than the sequences gathered from outside the clean room. While the CC and CC+ library cover over 80% of the hypothetical species diversity, only 40% of the calculated species variety is present in the UC clone library.

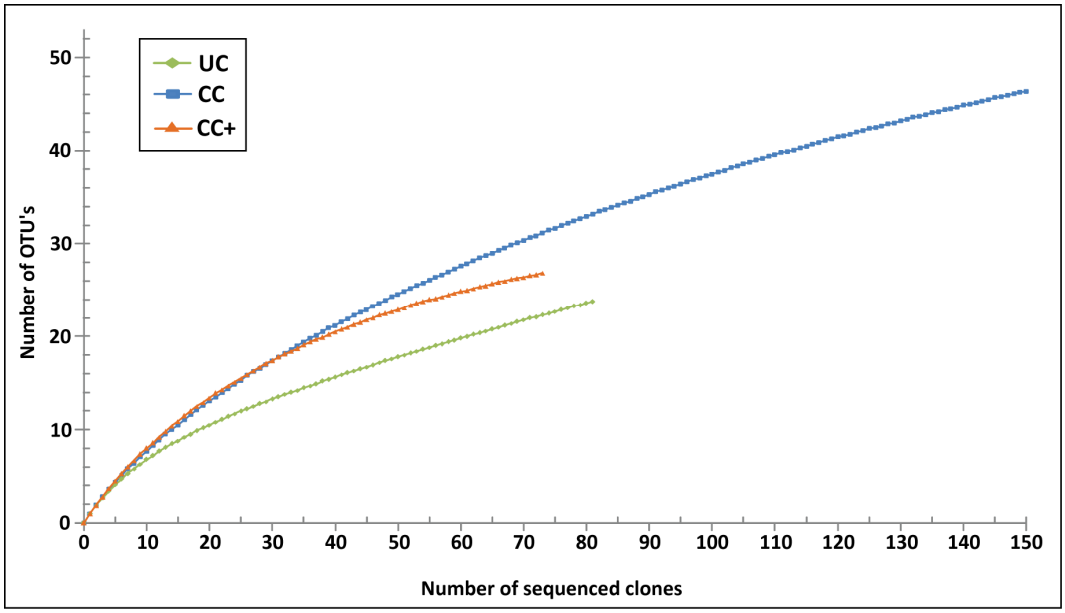

Figure 16: Rarefaction curves calculated for the different clone libraries
The green line represents the clone library from outside the clean room. The library exhibits a steeper slope indicating that the species diversity has not been fully sampled in this area. The curve for the CC library (inside the clean room = blue) begins to level off, indicating a relatively good species representation. The CC+ curve (inside the ATV = orange) illustrates an almost complete species coverage by the flattening of the rarefaction curve.

Table 13: Statistical analysis of the compiled 16S rDNA libraries from ESA's Hydra facility

	Area	Clones	OTU's	Singletons	Doubletons	C_{good}	S_{Chao1}	$S_{coverage}$
Library 1	**UC**	**142**	**24**	**15**	**2**	**89%**	**60**	**40%**
Library 2	**CC**	**404**	**58**	**19**	**14**	**84%**	**69**	**84%**
Library 3	**CC+**	**71**	**18**	**6**	**3**	**92%**	**22**	**82%**

3.4.4 Analysis of the uncultivable bacterial species diversity and distribution

3.4.4.1 Phylogenetic composition of uncultivable bacteria

The 16S rDNA inserts of the 745 successfully sequenced clones were compared to a database consisting of over 5,000 bacterial strain type sequences (Caltech Novel Technology Report #4478). Out of these clones, 415 could be identified with a certainty of above 97%, and within which 80 different species were recognized.

Over 93% of the individual species belonged to three mayor bacterial phyla. The *Firmicutes* are the most abundant phylum of the uncultivable bacteria. More than one third of the identified clones belong to this phylum (45%), which consists of the classes of *Bacilli* (33.75%) and *Clostridia* (11.25%). The second largest phylum are the *Proteobacteria* which constitute 30% of the sequenced clones. This phylum is represented by bacteria of the *Alpha-*, *Beta-*, and *Gamma-Proteobacteria* classes which, respectively, constitute 11.25%, 6.25% and 12.50% of the overall established clones. The *Actinobacteria* are the third mayor phylum amounting to 18.75% of the present uncultivable species. In addition, clones of the phyla of *Bacteroidetes, Cyanobacteria, Deinococcus-Thermus* and *Fuso-*

bacteria were found in ESA's Hydra facility amounting to the final 7% of the present uncultivable species. The exact number of clones found and their affiliation to the different phyla can be found in Table 14.

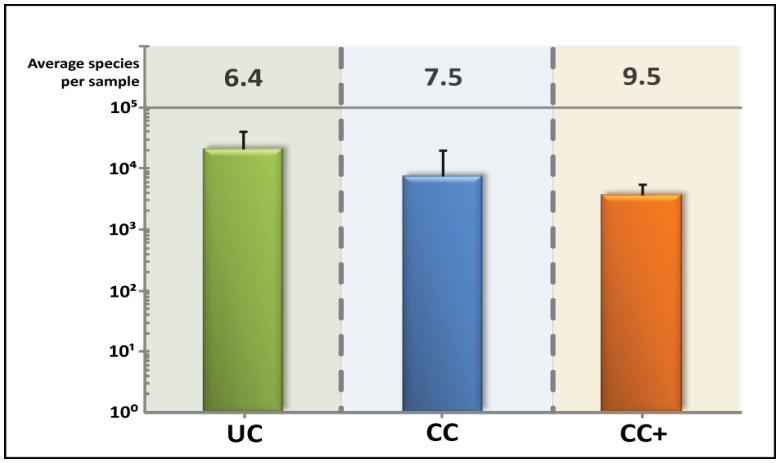

Figure 17: Average contamination level and detected average species per sample of the three distinct cleanliness zones inside ESA's Hydra facility
The colors represent the three cleanliness levels present inside ESA's Hydra facility: outside the clean room (UC = green), inside the clean room (CC = blue) and inside the highly access-restricted ATV (CC+ = orange).

Comparing the average bacterial species diversity between the three distinct access-restriction zones (UC, CC and CC+) it is noticeable that the number of identified species is reciprocal to the amount of cultivable bacteria present in the respective zone. Figure 17 highlights the relationship between the detected bacterial cfu present and the identified species for each cleanliness zones.

Neighbour joining trees were compiled to establish the phylogenetic relatedness of the identified bacteria by utilizing the ARB software package (www.arb-home.de). Therefore, phylogenetic trees of the cultivable (heterogeneous and heat tolerant isolates) and uncultivable bacterial fractions were constructed and are depicted in Figure 18.

More details about the identified uncultivable bacterial species can be found in Table 19 and 21 of the appendix, or on the Data-CD.

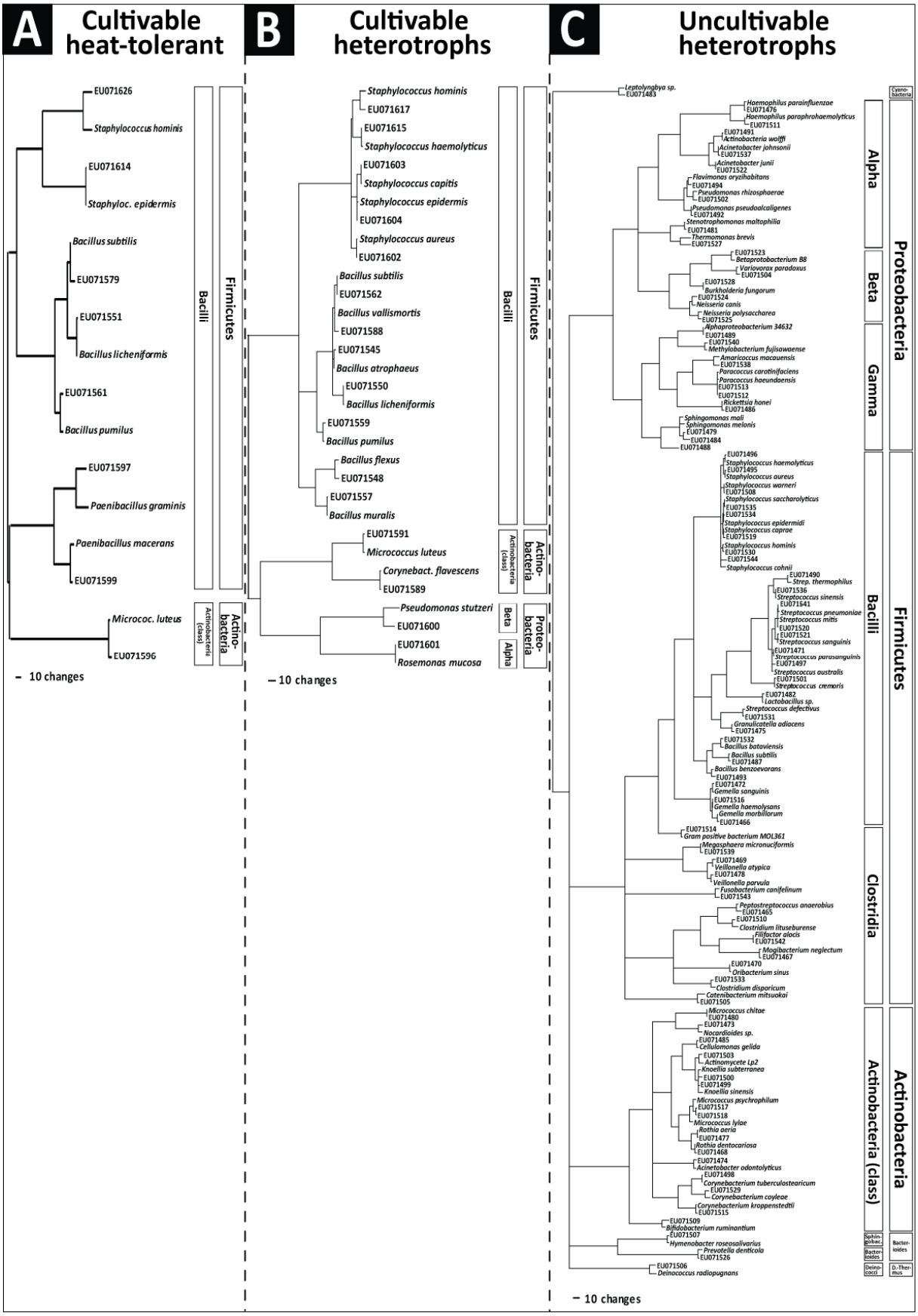

Figure 18: Phylogenetic trees (neighbour joining) of the identified (≥97% similarity) cultivable and uncultivable bacteria collected from ESA'S Hydra facility. Environmental clones are represented by their GenBank accession number, while named species represent the closest known bacterial species to the collected species. Larger versions of the phylogenetic tress and the distance-table can be found in Figure 27-29 of the appendix or as pdf-files on the Data-CD.

Table 14: Phylogenetic composition of the 16S rDNA-clones collected at ESA's Hydra space craft assembly & testing facility

Phylum/class	**Number of clones**	**% overall**
Firmicutes	**36**	**45.00%**
Bacilli	27	33.75%
Clostridia	09	11.25%
Proteobacteria	**24**	**30.00%**
Alpha-Proteobacteria	09	11.25%
Beta-Proteobacteria	05	6.25%
Gamma-Proteobacteria	10	12.50%
Actinobacteria	**15**	**18.75%**
Bacteroidetes	**02**	**2.50%**
Cyanobacteria	**01**	**1.25%**
Deinococcus-Thermus	**01**	**1.25%**
Fusobacteria	**01**	**1.25%**
Sum	**80**	**100%**

3.4.4.2 Distribution of uncultivable bacteria within the Hydra facility

After the phylogenetic composition of the uncultivable bacteria community was identified by 16S rDNA examination a species distribution analysis between the three established categories (UC, CC and CC+) was conducted.

Eighty-one percent of the identified species were only detected in one of the four different cleanliness areas. While 15% of the uncultivable bacteria could be detected within two categories, only three bacteria were present in all the created zones. These four species were *Granulicatella adiacens, Mycobacterium chitae*, *Streptococcus mitis* and *Streptococcus pneumoniae*. Figure 19 depicts the detected species distribution within the three different access-restriction zones inside ESA's Hydra facility.

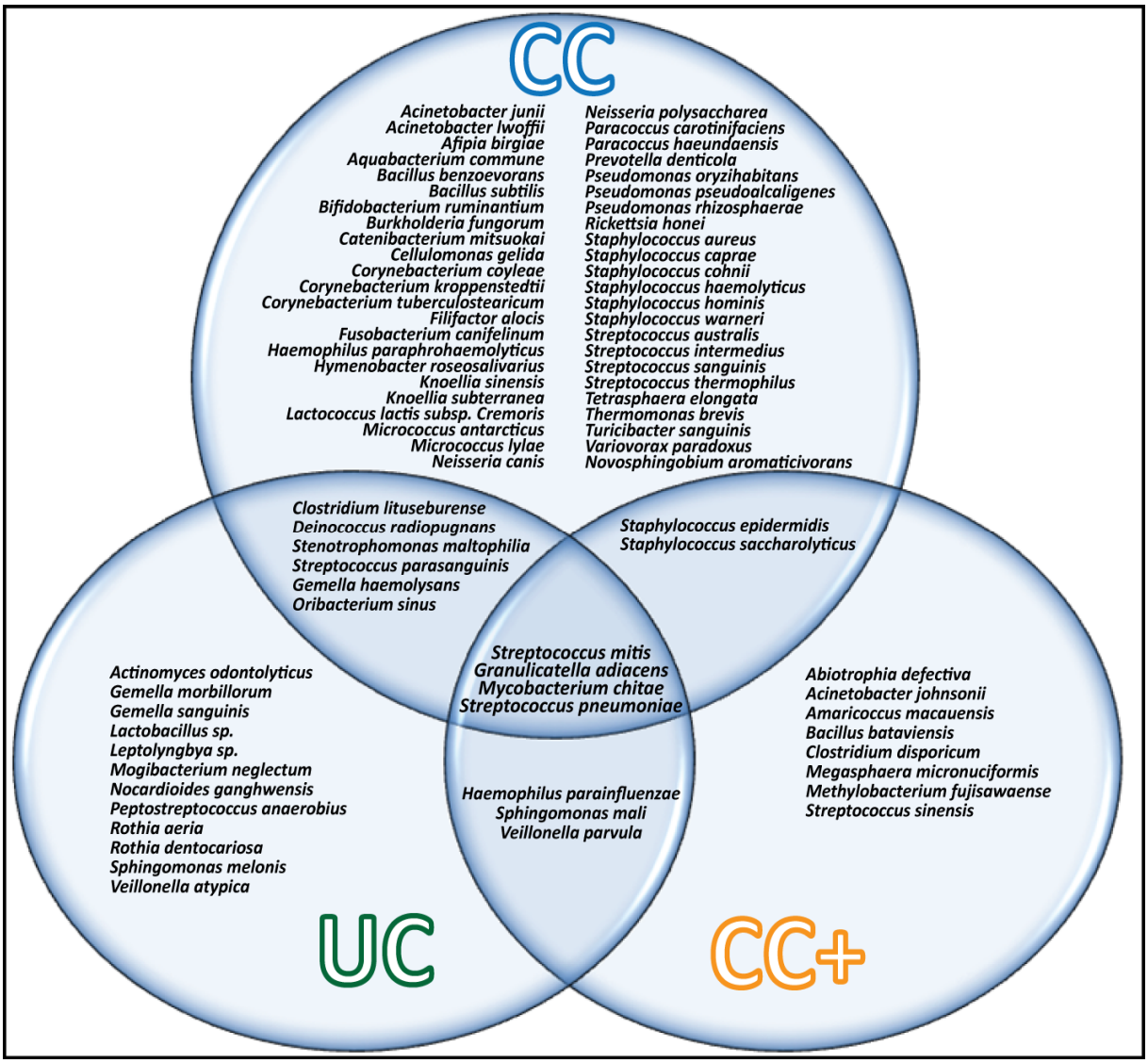

Figure 19: Uncultivable bacterial species distribution analysis

The Figure highlights the distribution pattern of the uncultivable bacteria within ESA's Hydra facility. The species were grouped into three zones representing the different cleanliness levels present inside ESA's Hydra facility: outside the clean room (UC = green), inside the clean room (CC = blue) and inside the highly access-restricted ATV (CC+ = orange).

3.5 Comparison of the detected cultivable and uncultivable bacteria diversity

In this study the bacterial composition of a class 100k clean room was surveyed and analyzed. Techniques for culture-based and molecular-based profiling of the bacterial community were therefore chosen to compile a realistic overview of the microorganisms prevalent inside the facility.

When comparing the variety of detected cultivable and uncultivable bacteria, it is noticeable, that only a fraction of the identified bacteria could be grown under laboratory conditions. When culture-based techniques were used to enrich the bacterial community before 16S rDNA sequencing, the diversity dropped perceptibly compared to the richness seen by direct molecular-based detection. While bacteria from 8 different phyla were recognized within the three 16S rDNA libraries, only 3 phyla could be identified after a cultivation phase was included. Furthermore, when comparing the

number of species detected by those two approaches, the difference becomes more pronounced. Figure 20 provides an overview over the species diversity detected by molecular-based and culture-based methods. Overall the culture dependent approach was only able to detect 16% of the number of individual bacteria verified by molecular-based profiling.

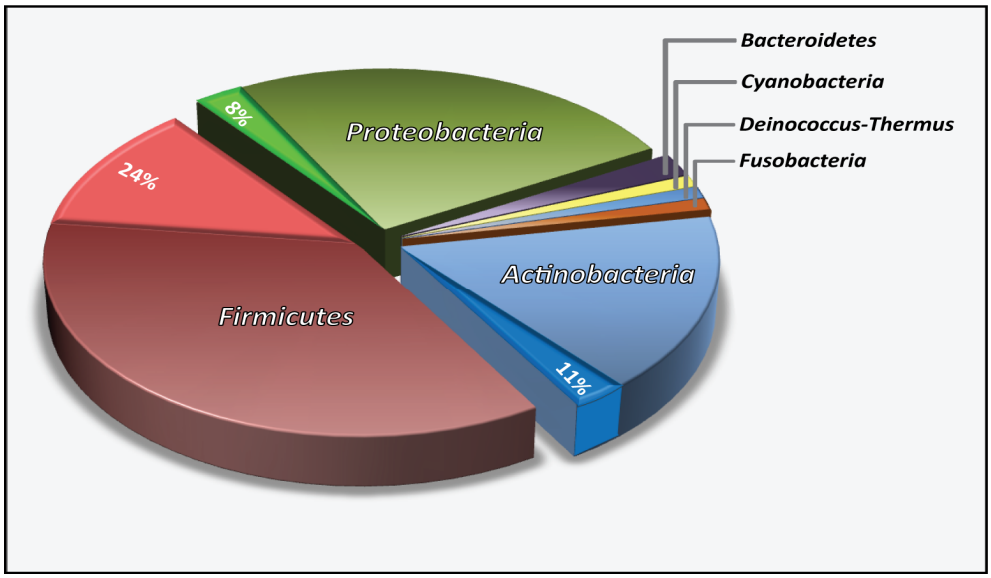

Figure 20: Species diversity of the Hydra facility
98 species were identified belonging to 8 different bacterial Phyla. The pie-chart represents the phyla com-position of the collected clean room bacterial community. The cultivable fraction of the *Actinobacteria* (11%) and *Firmicutes* (24%) and *Proteobacteria* (8%) phyla are represented by highlighted shades inside the corresponding (pie) sections.

Table 15: Habitat-association and gram-stain comparison between the identified cultivable and uncultivable bacteria fractions

Uncultivable bacteria			Cultivable bacteria	
Number of clones	**% overall**		**% overall**	**Number of clones**
		Gram stain		
31	39%	gram-negative	11%	2
49	61%	gram-positive	89%	16
80	100%		100%	18
		Habitat		
42	52%	human	39%	7
33	40%	environment	56%	10
5	6%	animal	0%	0
1	1%	insects	0%	0
1	1%	dairy products	5%	1
80	100%		100%	18

Most of the species identified in the cultivable, as well as in the uncultivable bacterial fraction can be linked to two major habitats: environmental sources and the normal human flora. While environmental sources represent the main bacterial contamination origin (56%) detected by cultivation-dependent methods, the human work crew is identified as the strongest polluting source for bacteria (52%) when molecular-based detection methods are applied. A small percentage of bacteria could also be traced back to animals, insects or dairy products. For exact details please refer to Table 15.

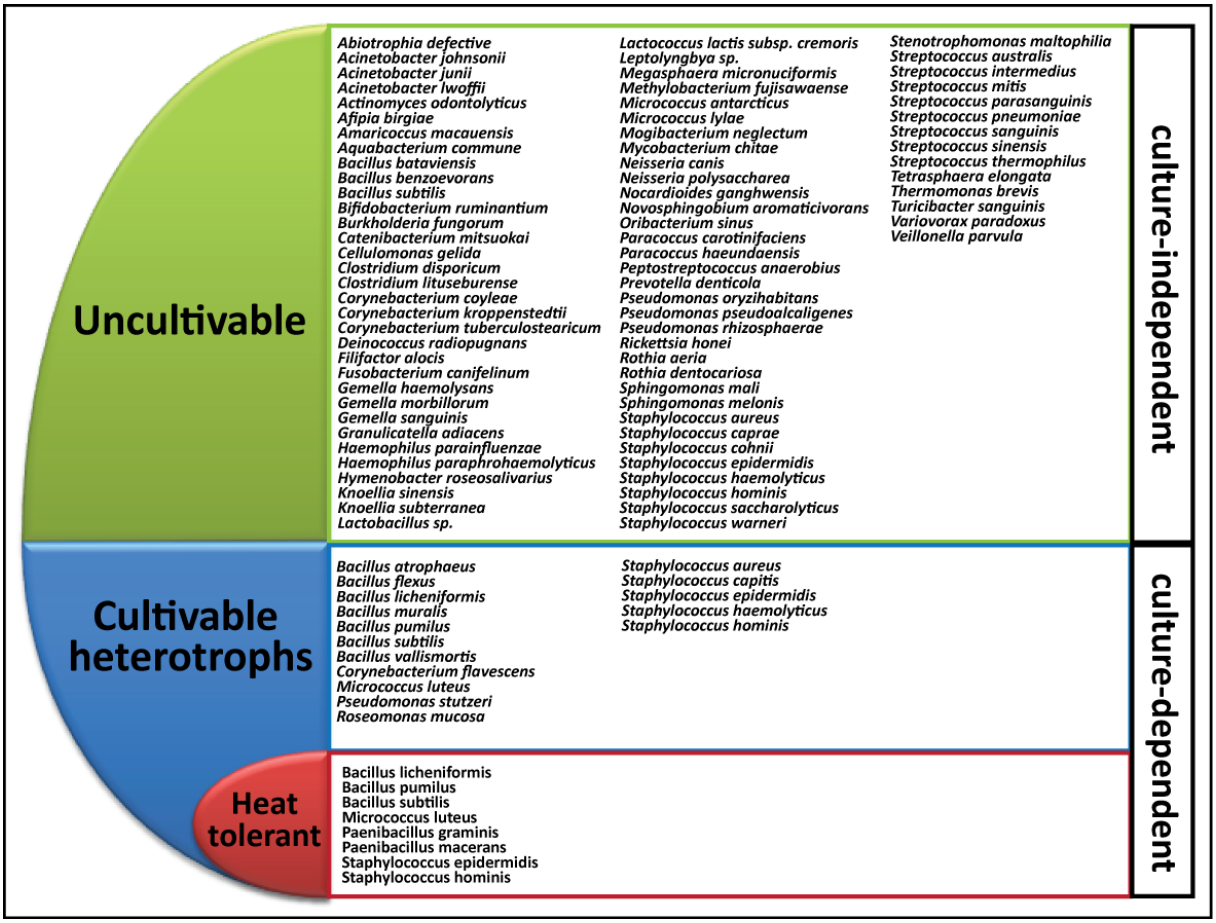

Figure 21: Identified cultivable & uncultivable bacterial species within ESA's Hydra facility

3.6 Resistance testing of the collected cultivable bacteria

Once the cultivable subset of the bacterial clean room community was identified and archived, tests were conducted to survey if this man-made "extreme environment" would lead to change in the physiological profile of the cultivable bacteria. For this reason the resistance of the isolated bacteria towards several harmful factors and conditions was tested.

3.6.1 Establishment of an efficient resistance screening procedure

Before testing the cultivatable bacterial community collected from the Hydra facility, a fast, flexible and reliable resistance screening method for a large number of diverse organisms needed to be established. The test scheme was selected to incorporate a range of different stress classes, while consisting of a "basic set up" which could be adapted to new agents as needed.
The test method was therefore mainly based upon the change in the absorption properties of the media due to the presence of grown bacteria. Resistance to the different stresses was defined as "lack of growth inhibition" by the agents. Depending on the kind of damaging influences tested, the organisms were either exposed before incubation (UV, desiccation) or grown in the presence of the harmful reagent (antibiotics, heavy metals, biocides, pH-shift and high salinity). The optical density of the samples was measured after an incubation period of 20h at 32°C.

By choosing the 96 well plate as base for this test, it was possible to accommodate negative, positive and background controls on each plate. This setup had the major advantage that the growth after/under the different stresses could be normalized, within each 96 well plate, to the actual maximal growth (untreated bacteria in standard medium). The so-generated plate specific but normalized results could then be compared between different bacteria species and 96 well plates. With the results of the first screening, a comprehensive resistance ranking system between the isolates of the community was established. The ranking system was constructed by comparing the relative growth of every isolate under each specific treatment within the collected community. The isolates were arranged by their ability to growth under the tested conditions and points were allocated for their relative place (one to n) within the tested community. The twenty strongest growers were given points as calculated by this equation:

Point value = 21 - relative place in the community ranking

Every isolate not in the group of the best twenty was given zero points for the appropriate test. Depending on the ranking within the cultivable bacterial faction each isolate was given zero to 21 points for every treatment. The points of the relevant treatment groups (antibiotics, heavy metals, biocides and environmental conditions) were added up and a community ranking established for each aspect.

From the 82 bacteria successfully isolated from the two sampling trips to the Hydra facility's clean room 62 were further chosen for resistance testing. The exact growth percentage of each bacterial strain under the different influences can be found in Table 22 of the appendix or, in more detail, on the Data-CD.

3.6.2 Antibiotic resistance

A spectrum of six antibiotics was tested on the bacteria isolated from ESA's Hydra facility. The applied antibiotics were selected for their ability to act upon different cellular targets, allowing the study of the isolates resistance behaviour towards several antibiotic inhibitory mechanisms.

Overall, the reaction of the 62 isolates on the "community level" towards the six treatments varied very strongly between the utilized antibiotics. The response ranged from a slight inhibitory effect on most of the community with penicillin to very lethal, inhibiting the growth of almost all tested isolates, when using gramicidin.

The highest antibiotic resistant bacterium was collected outside the controlled area (EU071564) and belongs to the genus of *Bacillus subtilis*.

While the bacterium exhibits no real pronounced resistance towards any single one of the tested antibiotics, the clone was able to grow moderately during all antibiotic treatments. A strain of *Staphylococcus epidermidis* (EU071607) also sampled outside the clean room exhibited the second highest resistance towards the tested antibiotics. The bacteria could withstand the inhibiting effects of penicillin and trimethropin effectively. While being able to grow moderately under the influence of nissin, the strain could not counter the harmful effect of rifamp, ciprofloflaxin or gramicidin.

Figure 22A depicts the combined resistance response of the cultivable bacterial fraction to the 6 tested antibiotics. The point-values were calculated for each isolated strain as described in 3.6.1 to identify noticeable individual strains within the tested cultivable bacterial fraction. As mentioned above, two strains exhibited raised survival abilities. The actual percentile growth of the two marked bacterial strains during the antibiotic exposure is depicted in Figure 22B & 22C. Each antibiotic was applied in a four log concentration range to identify the inhibitory antibiotic concentration in more detail.

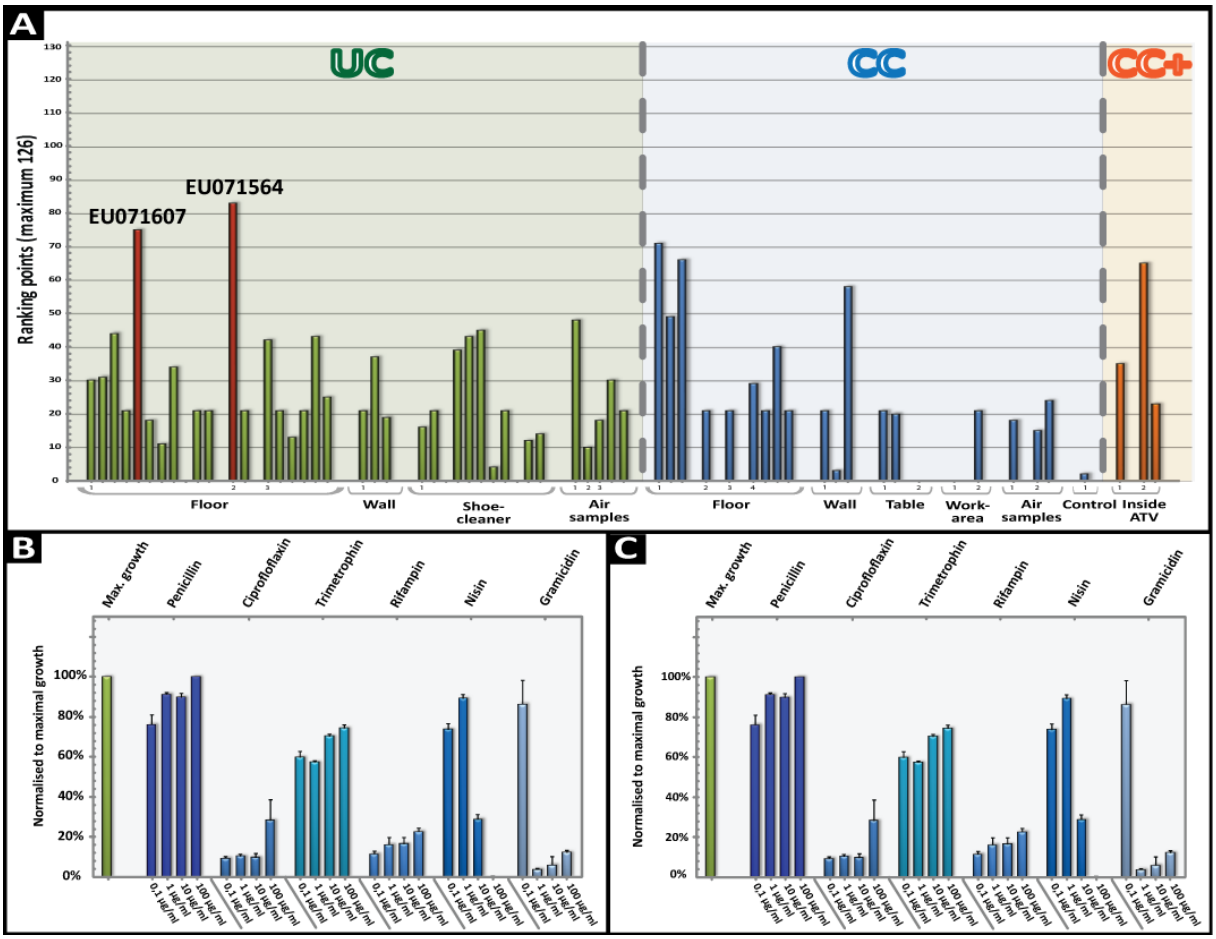

Figure 22: Antibiotic resistance of the collected bacterial isolates

Fig A: Ranking points of tested isolated bacterial strains. The isolates are grouped depending on the cleanliness zones from which they were collected: outside the clean room (UC = green), inside the clean room (CC = blue) and inside the highly access-restricted controlled ATV (CC+ = orange).

Fig B: Growth of the isolate EU071564 under the influence of the six tested antibiotics. Each antibiotic was tested in a 4log concentration range.

Fig C: Growth of the isolate EU071607 under the influence of the six tested antibiotics. Each antibiotic was tested in a 4log concentration range.

3.6.3 Heavy metal resistance

The isolates were tested for their ability to grow under the influence of six different heavy metals. The chosen heavy metals ranged from rare, highly toxic metals like mercury to metals which are commonly found naturally in the environment like copper.

Though the different inhibitory quality of the heavy metals is not noticeable at the highest doses used (100 mM or, respectively, 10 mM for mercury), the varying inhibitory severity becomes quite evident at lower concentrations.

During the screening no community level resistance towards any heavy metal was discernable; only two of the tested isolates distinguish themselves by their ability to survive the damaging effect of the heavy metals [Figure 23A].

A clone belonging to the strain of *Staphylococcus haemolyticus* collected from inside the clean room (EU071615) was able to grow under the influence of most of the tested heavy metals. The clone was

able to grow moderately in the presence of cobalt, copper and cadmium, and exhibits an almost unhindered ability to grow in the presence of arsenic acid and zinc. The only exception was mercury which was able to inhibit the clone at all the tested concentrations.

The second organism able to grow while being exposed to the selected heavy metals was found outside the restricted area (EU071564) and identified as *Bacillus subtilis*. Even though the strain did not exhibit any particular strong resistance during the tests, it was able to grow moderately under the influence of the applied heavy metals. This strain of *Bacillus subitilis* did previously stand out for its ability to grow moderately during the antibiotic treatments.

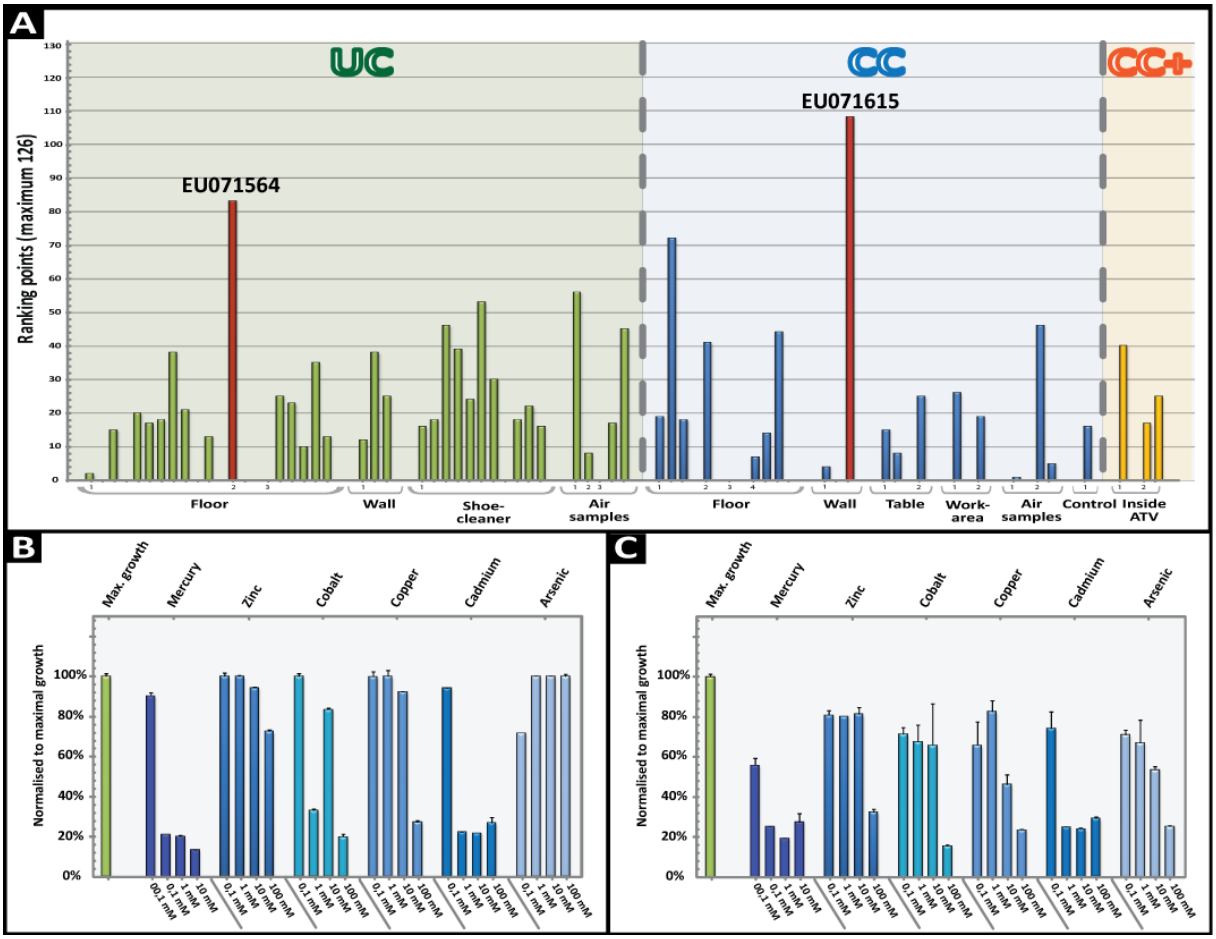

Figure 23: Heavy metal resistance of the collected bacterial isolates

Fig A: Ranking points of tested isolated bacterial strains. The isolates are grouped depending on the cleanliness zones from which they were collected: outside the clean room (UC = green), inside the clean room (CC = blue) and inside the highly access-restricted controlled ATV (CC+ = orange).

Fig B: Growth of the isolate EU071615 under the influence of the six tested heavy metals. Each heavy metal was tested in a 4log concentration range.

Fig C: Growth of the isolate EU071564 under the influence of the six tested heavy metals. Each heavy metal was tested in a 4log concentration range.

3.6.4 Biocide resistance

Biocides are defined as "chemical substances which are able to inhibit the growth or kill different forms of living organisms". The 62 chosen isolates from Hydra facility community were subjected to the following biocidal treatments to analyze the effectiveness of different cleaning agents and procedures on this isolated bacterial community. For the class of alcohols the isolates were subjected to 50% (v/v) of Ethanol and Isopropanol. Formaldehyde (8%) (v/v), Glutaraldehyde (1%) (v/v) were chosen to represent the effect of the class of aldehyds on the bacterial community. A five percent (v/v) solution of H_2O_2 was chosen as representative reagent for the class of radical producer. The effect of UV-C irradiation of up to 2000 J/m^2 was furthermore tested as this kind of radiation is often used in controlled areas for surface and air sterilisation.

The entire set of biocides tested had a strong impact on the community. Almost all isolates were completely inhibited in their growth by the used biocidal compounds, and only the damaging effect of H_2O_2 and UV was survived by some of the organisms.

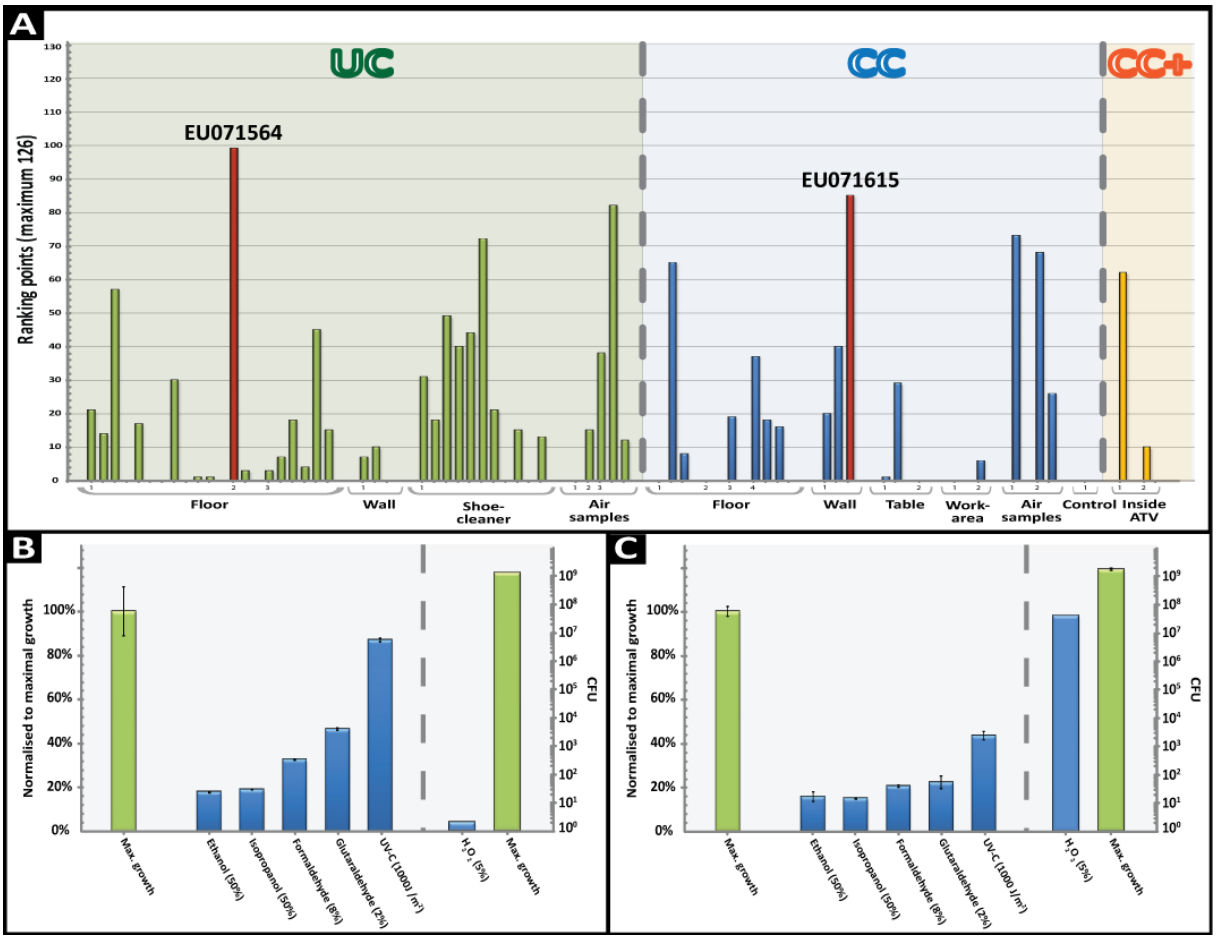

Figure 24: Biocide resistance of the collected bacterial isolates

Fig A: Ranking points of tested isolated bacterial strains. The isolates are grouped depending on the cleanliness zones from which they were collected: outside the clean room (UC = green), inside the clean room (CC = blue) and inside the highly access-restricted controlled ATV (CC+ = orange).

Fig B: Growth of the isolate EU071564 under the influence of the six tested biocides.

Fig C: Growth of the isolate EU071615 under the influence of the six tested biocides.

The strain of *Bacillus subtilis*, collected from outside the restricted area (EU071564) was once more one of the strongest survivors under the tested conditions. It was able to exhibit minor growth in the presence of the different biocidal compounds (ethanol, isopropanol, formaldehyde and glutar-dialdehyde) as well as the damaging effect of the UV treatment. It should be noted that this is the same strain which was able to moderately survive the damaging effect of the tested antibiotics and heavy metals.

Another strain which was able to survive the effect of the biocides to some extent belonged to the genus of *Staphylococcus haemolyticus* and was found inside the class 100k clean room (EU071615). The strain was able to exhibit minor growth during the presence of the biocidal components and showed almost unhindered growth after the H_2O_2 treatment. Again this strain was noticed before in [3.6.3] for its resistance to the damaging effect of the tested heavy metal.

3.6.5 Environmental conditions resistance

Different treatments representing extreme environmental conditions, were chosen to inspect the adaptability of the 62 isolates towards ecological stresses. The parameters included: Alkaline (ph 9.5) and acidic (pH 4) pH, high salinity (1 M NaCL), heat shock (80°C, 15 min) as well as a desiccation period of seven days.

For each of the different treatments several of the organisms collected were able to grow under a single tested stressful condition, while two of the tested bacteria should be noted for their ability survive under several of the tested harmful parameters.

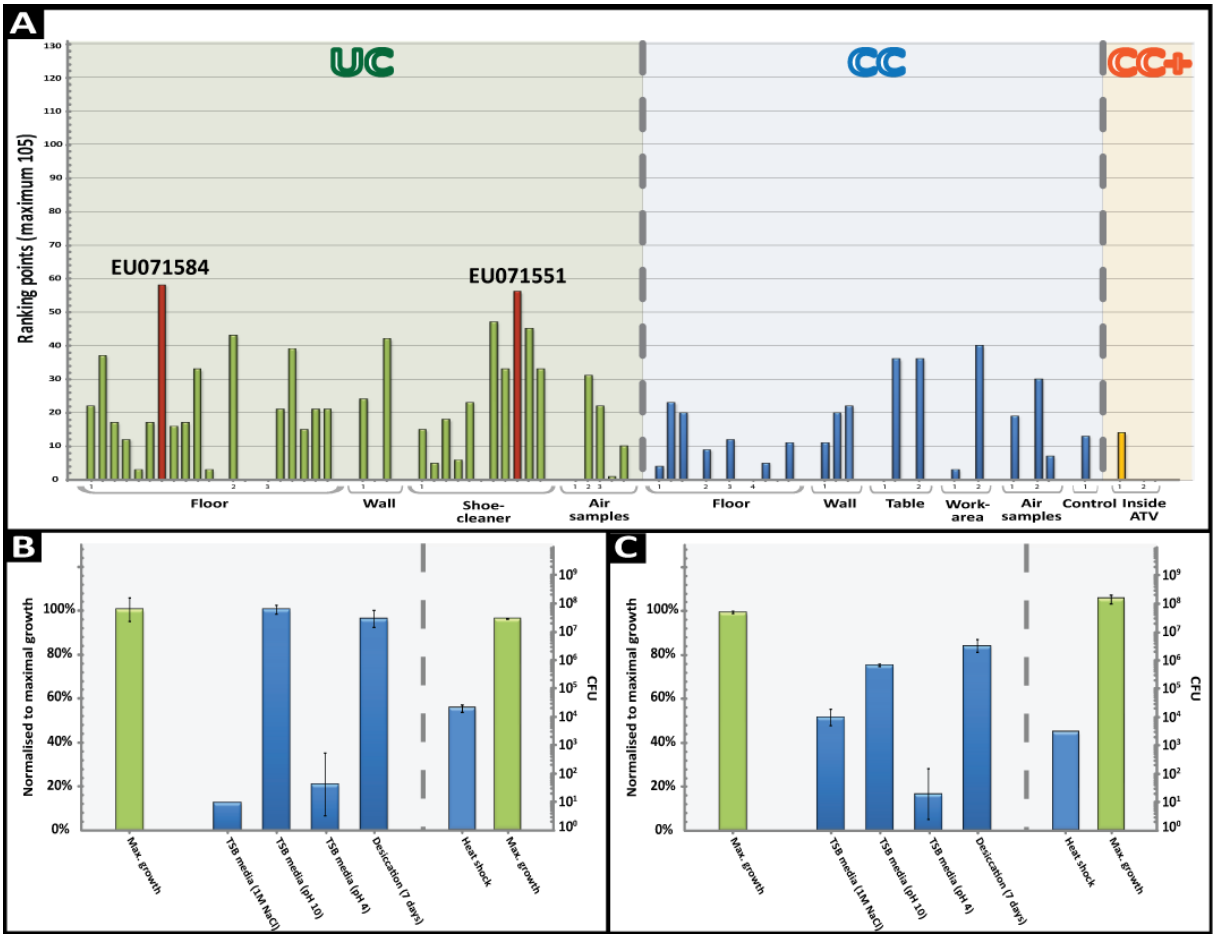

Figure 25: Environmental factors resistance of the collected bacterial isolates
Fig A: Ranking points of tested isolated bacterial strains. The isolates are grouped depending on the cleanliness zones from which they were collected: outside the clean room (UC = green), inside the clean room (CC = blue) and inside the highly access-restricted controlled ATV (CC+ = orange).
Fig B: Growth of the isolate EU071551 under the influence of the tested environmental stresses.
Fig C: Growth of the isolate EU071584 under the influence of the tested environmental stresses.

A strain of the bacterial genus *Bacillus licheniformis* isolated from the shoe-cleaner outside the class 100k clean room (EU071551) was able to grow almost uninhibited at pH of 10 or in medium containing 1 M NaCL. The clone exhibited furthermore a strong ability to withstand the desiccation stress.

The strain (EU071584) identified as a *Bacillus subtilis* strain, also gathered from outside the clean room, is the second noticeable organism. The strain was able to grow at nearly normal rate at pH 10 as well as survive the desiccation and heat shock treatments.

3.6.6 Resistance comparison between strains of one species

The 62 tested isolates were identified by 16S rDNA analyses as described in 3.6. Overall, 18 different bacterial species were identified which could be assigned to the phyla of *Actinobacteria* and *Firmicutes*. The experimental design [see 2.4] for the resistance tests was created to be able to screen a large number of organisms. This setup made it possible to test several strains of each of the isolated

bacterial species for variations in their resistance behaviour. Most of the bacterial strains collected from different areas inside the Hydra facility exhibit species specific resistance characteristics. None the less, several individual strains were noticed for their exceptional resistance potential.

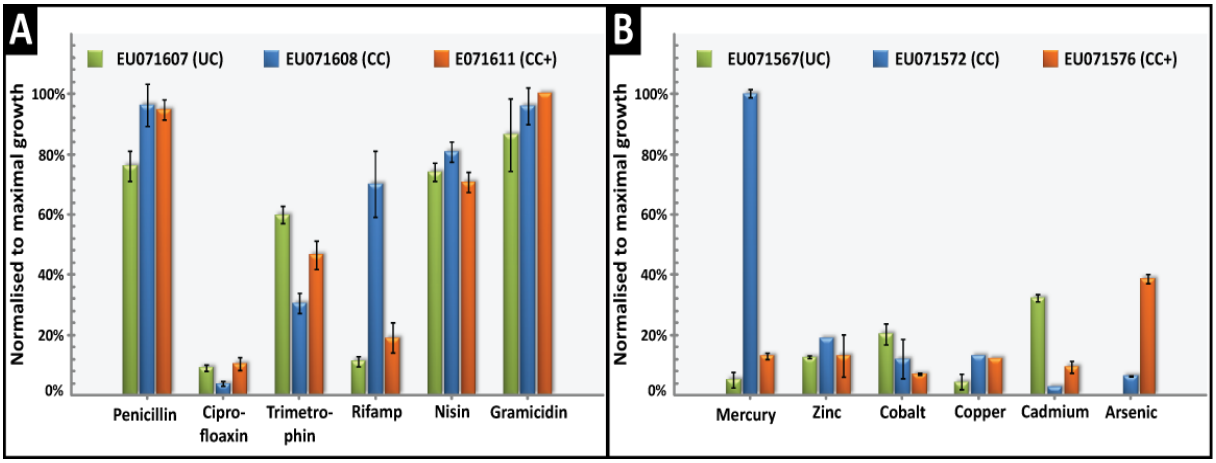

Figure 26: Variations in the resistance characteristics of individual strains collected at ESA's Hydra facility

The colour of the isolates represents the area the strain was collected from: outside the clean room (UC = green), inside the clean room (CC = blue) and inside the highly access-restricted controlled ATV (CC+ = orange).
A: Variation of the antibiotic resistance of 3 strains identified as *Staphylococcus epidermidis*
B: Variation of the heavy metal resistance of 3 strains identified as *Bacillus subtilis DSM10*

When comparing the antibiotic resistance of three bacterial strains all identified as *Staphylococcus epidermidis* by 16S rDNA and morphology analysis, one clone (EU071608) exhibited a strongly raised resistance against the antibiotic rifampin [Figure 26A]. The three strains were collected from different sample areas: one strain was found outside the clean room (EU071607) while the two other strains were collected from inside (EU071608 & EU071611) the restricted area. This increased resistance is not shared by the other strains of the species nor is it within the standard variation of the experimental setup.

Similar observations were made when comparing the other tested resistances amongst the different strains. For example, as is depicted in Figure 26B, a strain identified as *Bacillus subtilis* exhibited a markedly raised resistance against mercury while its ability to withstand the harmful effect of arsenic acid was decreased.

The strain specific analysis of the cultivable bacterial resistance characteristics revealed several differences between the treatment responses of individual strains within one species. Thus, for this environment, species-wide changes in the resistance characteristics could not be detected in this study but several individual, strain specific, alterations were noticed.

IV. Discussion

The analysis of environmental samples taken from controlled areas like clean rooms or operation theatres is a difficult task due to the low amount of biomass present. Most of the standard survey strategies so far include cultivation and enumeration steps which will encompass only a minor fraction of the community of microorganisms actually present at those sites. The integration of new, molecular-based methods into the established standard protocols for biological contamination monitoring of clean rooms offers the possibility to gather a more realistic estimation of the bacterial species present in such “man-made” extreme environments.

During this study it was possible to enhance the standard contamination assessment procedure of clean rooms by including molecular based techniques into the sample analysis setup. This change made it possible to study the cultivable and uncultivable fraction of the bacterial clean room community. During the project 82 different cultivable bacteria strains could be isolated and 80 different uncultivable bacterial species could be identified by 16S rDNA analysis. Furthermore, it was possible to compile the resistance profiles of over 60 bacterial strains isolated from inside the facility.

4.1 Analysis of the standard NASA clean room sampling procedure

The initial aim of this study was the optimization of clean room sampling through the integration of new molecular techniques. As mentioned in 1.2, most techniques used to survey bacterial con-tamination in industrial clean rooms were developed over 30 years ago. Although they were refined over time, they still contain several drawbacks and impairments. When deploying swab techniques for surface sampling the results are not as reproducible as, for example, using techniques like contact plates (Tidswell, 2005; Van Loosdrecht *et al.*, 1990). This is due to the fact that sampling conditions can vary highly during field trips and that swab handling is individual for each operator (e.g. pressure, speed, exact angle etc.) (Baldock 1974; Favero *et al.*, 1968).

In this study it was demonstrated that using NASA’s standard microbial swab sample processing method (Nasa NPG: 5340.1D) only in average 50% of the actual surface bacteria present were taken up during swabbing. From these collected bacteria again almost 60% were retained in the swab during sample processing. These effects lead to an overall underestimation of the bacterial con-tamination by almost 300% (Nellen *et al.*, 2006). A result which is within the range of other studies assessing sampling procedures using cotton swabs (Angelotti and Foter, 1958a; Angelotti *et al.*, 1958b). Though no major difference in the sampling characteristics of the tested cotton and rayon swabs could be detected in this study [3.1.1.1], there are several different swab-types in use today which exhibit diverse specific properties (Buttner *et al.*, 2004; Favero *et al.*, 1968; Rose *et al.*, 2004).

All the introduced sampling techniques rely heavily on cultivation processes like isolation or enrichment cultures (Puelo *et al.*, 1977; Tidswell, 2005) to detect possible bacterial contamination. As discussed in 1.3, the inability to cultivate most of the known bacterial species in the laboratory today will permit only a minor fraction of the actual present community to be detected by these methods (Amann et al., 1995). Therefore, cultivation independent detection methods need to be integrated into the overall survey setup to obtain a more precise impression of the actual present microbial community (Head *et al.*, 1998).

Once it was evident that over 50% of the collected bacteria were retained in the swab [3.1.1.2], this opportunity was taken to incorporate molecular-based methods into the standard sampling process. In this study, a method was adapted and refined to extract DNA directly from the sampling swabs utilizing the retained bacterial fraction to conduct culture-independent phylogenetic diversity studies. This approach provides the opportunity to analyze the cultivable and uncultivable fraction of the same swab, i.e. the same sample.

The procedure established in this study was created to be a robust technique which disrupts the cell integrity of sturdy organisms or cell forms like gram-positive bacteria or spores. Therefore, several cell lysis approaches were united to efficiently but "gently" break open the bacterial cells while keeping DNA damage at a minimum. It was possible to obtain PCR grade DNA for 16S rDNA sequencing from as low as 10^2 spores [3.1.2.1].

To identify the most appropriate method for DNA purification two kits utilizing different DNA binding methods were compared against a commonly used phenol-chloroform based DNA purification technique (Ogram *et al.*, 1995; Selenka and Klingmüller, 1991; Tebbe and Vahjen, 1993; Tsai and Olsen 1991) [3.1.2.2]. While both kits were able to extract PCR grade DNA from as low as 10^2 cells, Qiagen QIAamp DNA Micro Kit was chosen as standard DNA purification solution on account of its higher reproducible results.

Using the newly established protocol to handle the samples taken at ESA's clean room [3.2], high quality, PCR grade DNA could be obtained from each collected sample. Over 92% (745/808) of the created clones could be assembled and analysed further. Amplicons of 8% (63/808) of the clones could not be assigned to a species as a result of artefacts in the amplification and cloning process. This confirms the high quality and genomic diversity of the clean room DNA obtained in this study and provides a solid basis for the phenotypic analysis.

4.2 Cultivation dependent bacteria: occurrence and diversity

In order to confirm the increase in diversity detectable through molecular analysis, the diversity and level of contamination of the facility had to be determined using the standard NASA protocols. 18 different areas within the Hydra facility were sampled and depending on their access-restriction and "cleanliness level" subdivided into three distinct zones. The changing area outside the clean room represents the basic, uncontrolled environment (UC) inside the Hydra facility. The next "cleaner" level (CC) is the clean room itself which can only be entered after changing into specialised clothing and observing class 100k clean room procedures (ISO 14644-1). The ATV-module inside the clean room represents the maximal restricted area (CC+) which can only be entered by one person at a time wearing a full body suit. Though this area is not fully separated from the clean room (no barrier prevents the air from being interchanged between the two compartments), the access-limitations minimizes the chance of contamination by the workforce.

Using a slightly modified standard NASA's culture depended methods (Nasa NPG: 5340.1D, but R2A as medium and no heat shock) it was possible to detect the heterotrophic cultivable bacterial community inside the Hydra facility. The range of detected bacteria did vary between sample locations, but a constant decrease in the cultivable cell number in correlation with the implemented restriction and control level was evident [Figure 17]. Overall a contamination decrease by 90% between the uncontrolled (UC) and highly controlled (CC+) area was observed. Though it was possible to verify the existence of bacteria in all of the three distinct cleanliness levels, the average colonies detected per sample dropped from 8 colonies (UC) to two colonies per site inside the clean room.

The bacterial cell count of the heat tolerant cultivable fraction is about a factor 10 lower than the cell count for the untreated cultivable clean room population [3.4.5.1]. Due to the decrease in the cell count of heat tolerant bacteria below the average of one colony per 25cm^2, no meaningful comparison of the bacterial distribution between the inside and outside of the restricted area can be conducted.

Several studies (La Duc *et al.*, 2007; Puelo et al, 1973) support these results and highlight the fact that the restriction level deployed inside a class 100k clean room will be sufficient to lower the overall particle count and amount of cultivable bacteria but are not sufficient to prevent micro-organisms from entering. The reduction in the detected cultivable bacterial colonies is most likely caused by the stricter, growth-suppressing, environmental conditions inside the clean room and not due to the decrease in the amount of present bacteria. This theory is supported by the results of the uncultivable bacterial diversity analyzed during this survey [3.4]. Using culture-independent techniques it was possible to detect bacterial DNA in all the collected samples from the Hydra facility.

Therefore, it is likely that microorganisms will constantly be carried into the clean room by the human workforce; but due to the harsh conditions inside the restricted area, only a fraction of the bacteria will be able to survive and grow. This, in turn, will lead to a decrease in the cultivable bacterial count but not in the culture-independent tally.

4.2.1 Contamination analysis of the Hydra facility

One goal of this study was the enumeration and cultivation of the bacterial community present inside ESA's Hydra facility. From the collected samples it was possible to isolate and analyse 82 bacterial strains. The 16s rDNA analysis of these pure cultures revealed a total of 18 different species within the collected cultivable community. This fraction was dominated by members of three phyla: the *Firmicutes* (78%), the *Actinobacteria* (11%) and the *Proteobacteria* (11%). During the 1960's and 70s several cultivation based studies identified the *Actinobacteria* and *Firmicutes* as the dominant bacterial phyla present inside the tested spacecraft assembly clean rooms (Puleo *et al.*, 1967; Puelo *et al.*, 1977). More than 90% of the overall detected cultivable species were affiliated with these two phyla, a fact also seen in this study. Favero and colleagues were able to demonstrate that under the applied, specific cultivation parameters this microbial composition is not only present in specialised i.e. space assembly clean room facilities, but is also common for man-made controlled habitats (Favero *et al.*, 1966; Favero *et al.*, 1968).

Even though several studies were successful in creating experimental setups and cultivation conditions which allow the isolation of new species from heterogeneous environments (Hugenholtz, 2002; Leadbetter, 2003; Zengler *et al.*, 2002), the effort required to generate these experimental setups is exceedingly high and the extra species isolated are still only a minor fraction of the complete diversity. This limited variety within the cultivable bacteria species was also seen within the analyzed clean room samples. The number of detected bacterial species from the cultivated fraction (18 species) was almost five times lower than the number of species identified from the direct DNA extraction (80 species). This ratio is observed in other bacterial community of controlled environments like e.g. activated sludge (Wagner *et al.*, 1993). Both condition sets seem limit the species diversity in favor of sturdy, adaptable bacterial species which can survive and grow under stressed and selective conditions.

In addition three mayor bacterial species were evident and identified as members of the principal occurring phylum, the *Firmicutes*.

Strains of the *Bacillus subtilis* species were found in 80% of the collected samples and, thereby, represent the most frequent cultivable species identified in this study. The second and third strongest species *Staphylococcus epidermidis* and *Staphylococcus hominis*, were cultivable from 44%

and 39% of the sampled locations, respectively. Though these three species amount to 43% of all the collected cultivable bacteria, they represent only a minor fraction of the actual present bacterial diversity as it is detected by cultivation independent methods [3.5]. Therefore, it seems that the strong presence of these three bacteria is caused by a cultivation dependent overrepresentation rather than an actual dominance of the species.

ESA's MiDiv study (Rettberg *et al.*, 2006) applied a multitude of different incubation conditions to partly circumvent the bias generated by applying specific culturing conditions. The original samples were divided into various fractions and grown in 20 different conditions (pH, temperature, aerobe, anaerobe etc.) This concept increases the chance that the appropriate cultivation condition needed for specialised and rare bacteria will be included into the test setup. On the other hand, when this approach is used for such a low biomass environment, it may also create problems. By diluting the samples before incubation the number of bacteria in each sample might fall below the detection limit and lead to false negative results. Secondly, by including a broad range of culturing conditions, the chance that a bacterial species is actually incubated under suitable conditions decreases in accordance with the number of tested conditions.
Therefore, in this study only two media were used for the culturing of the collected bacteria. R2A-medium, a low nutrient content medium first introduced in 1985 by D.J. Reasoner (Reasoner and Geldreich, 1985), was used to permit a wide spectrum of slow-growing bacteria to develop without being suppressed by occurring fast-growing microbes. Secondly, to complying with NASA's bioburden sampling procedures (Nasa NPG: 5340.1D) of clean rooms, the second medium (TSB) was used for the cultivation of the heat shocked bacterial fraction to detect spore formers.

4.2.2 Analysis of the cultivable bacterial phylogenetic diversity

Since the large percentage of uncultivable bacteria in ecological samples became evident in the 1990's, direct DNA analysis of environmental samples developed into a commonly applied investigation approach for such samples (Hugenholtz *et al.*, 1998b; Sogin *et al.*, 2006; Torsvik, *et al.*, 1990). After the analysis of the sample taking process, it was possible to include cultivation independent techniques into the sampling protocols without the need to split the sample into multiple fractions [3.1]. This essentially made it possible to analyze each sample by direct DNA analysis as well as by standard microbial methods.

It should be noted that, the incorporation of the DNA based phylogenetic analysis methodology into the sample setup is a substantial enhancement. However, due to the intrinsic biases created by the applied techniques [1.4], this improvement will not lead to a full representation of the actual bacterial phylogenetic diversity present in the sampled environment.

4.3 Cultivation independent bacteria: diversity and distribution

The 18 sampled areas within the Hydra facility were subdivided into three "restriction zones" as described in [3.2.2]. Discrete clone libraries were created [3.4.3.3] from these zones to analyze the uncultivable phylogenetic diversity of each area.

4.3.1 Validation of the environmental 16S rDNA clone libraries

The sequence distribution was analyzed using the Good's coverage equation to determine if the clone libraries sufficiently represent the bacteria present in each sample. Based on this statistic, the libraries cover over 92% of the present sequences [Table 13], indicating that less than 10 new bacterial species would be identified for every 100 additional analyzed clones. This level of coverage signifies that the majority of uncultivable bacteria present within the samples were identified.
Rarefactions curves were calculated for each of the three mentioned groups (UC, CC and CC+). Whereas, the rarefaction curve from within the ATV (CC+) is leveling out, indicating a relatively complete sampling of the area's biodiversity; the rarefaction curve calculated from inside the clean room (CC) is still rising, suggesting a larger, yet incompletely, sampled bacterial community. Rarefaction analysis of the uncontrolled area (UC) also suggests that the bacterial phylotype richness could not be fully covered during this study and needs to be analyzed further.

The evidence given by the rarefaction analysis is further confirmed by the assessment of the probable total number of calculated phylotypes using Chao's phylotype richness estimator (Chao, 1984). The equation offers an unbiased estimation of the probable total number of different phylotypes present in a tested environment. The computed maximal phylotypes for the UC, CC and CC+ regions are respectively 52, 69 and 23 different species. The overall detection coverage can be calculated by dividing the actual identified species through the theoretically calculated maximal number of present bacteria ($S_{observed}/S_{max}$). The phylotype coverage of the tested three groups is 40% (UC), 82% (CC) and 84% (CC+) of the maximal calculated phylotype diversity.

The noticed large discrepancy between the bacterial community coverage outside (40%) and inside (≥ 81%) the clean room is likely caused by the difference in the environmental conditions present in these two areas. In, harsh, nutrient-poor surroundings (e.g. the clean room), slow-growing bacteria are able endure and survive without the risk of being out-competed by comparatively poorly adapted but fast-growing adversaries. This hypothesis would postulate a reciprocally correlation between the number of present bacteria and the species diversity, which can be observed in this study. Comparing the number of cfu's collected from outside the restricted area (10^3 cfu/m^2) to those inside the clean room (10^2 cfu/m^2) and the ATV (10^2 cfu/m^2) a correlation between the decrease in the number of present bacteria and the implemented cleanliness level was identified. At the same time, an increase

in the average number of species per area can be observed. Whereas 6 species outside the clean room and 8.5 species inside the restricted area could be identified, it was possible to collect 9.5 species per sample from inside the ATV [Figure 17]. The same relationship was observed in caves in Arizona where several areas inside a pristine and unused natural cave were opened for tourism. The bacterial contamination by the tourists was monitored and a reciprocal correlation between bacterial numbers and species diversity was recognized for differently accessible caves (Ikner *et al.*, 2006).

4.3.2 Analysis of the uncultivable bacterial phylogenetic diversity

Once the validity of the 16S rDNA libraries was confirmed, the phylogenetic composition of the uncultivable bacterial community was analyzed by sequencing and classifying over 800 16S rDNA clones. Of the analyzed clones, 65% exhibited a sequence similarity of over 97% allowing the identification of 80 different bacterial species within the cultivation independent bacterial community. The unmatched analyzed clones (35%) can be affiliated with 10 different phyla, two of which are candidate phyla without cultivated representatives (data not shown). The clones possess a sequence consensus of 83% to 96.9% similarity thereby, most likely, representing new uncharacterized bacterial species.

As observed with the cultivation-dependent microbial fraction, the bacterial species composition is dominated by few phyla. Of the 80 fully classified clones, 45% are associated with the *Firmicutes* phylum. The second strongest phylum is represented by the *Proteobacteria* contributing almost one third (30%) of all the identified species. The last mayor phylum, the *Actinobacteria*, still encompasses 18.75% of the detected organisms. Overall, more than 90% of the identified uncultivable bacterial species can be grouped into these three phyla. While a variety of bacteria belonging to the *Firmicutes* and *Actinobacteria* phyla could be detected by cultivation dependent methods, only 2 *Proteobacteria* species could be grown in the laboratory [3.3.1]. These results mirror the cultivation dependent sampling results of Puelo *et al.* who assayed the clean rooms and Apollo (1973) as well as Viking spacecrafts (1977) during their assembly phase. A study by Mitsui *et al.* (1997) was able to prove that α-*Proteobacteria* do grow, but rather slowly, on low nutrient media. In light of this data it is possible, even though a low nutrient medium (R2A) was used in study, that the applied cultivation conditions still selected for flexible fast-growing bacteria.

The ***Firmicutes*** phylum is composed of mainly aerobic, gram-positive bacteria and was frequently observed in the cultivation-dependent (78%) and -independent (45%) bacterial fraction. Members of both, the *Bacillus* and the *Clostridium* class, possess the ability to form endospores, enabling them to survive under harsh conditions like those present inside a clean room.
Among the detected species are typical representatives of *Firmicutes* normally found in environmental samples or with humans like *Bacillus subtilis* or *Staphylococcus epidermidis* (Madigan *et al.*, 2003). In addition to these normally innocuous bacterial species, several opportunistic pathogens like *Staphylococcus caprae* (dermatitis; Allignet *et al.*, 2001), *Abiotrophia defectiva* (infective endocarditis; Ohara-Nemoto *et al.*, 1997; Tart and van de Rijn, 1991) *Staphylococcus aureus* (wide range of infections; Archer, 1998) and *Streptococcus mitis* (endocarditis ; Hall and Baddour, 2002) as well as a significant pathogenic bacteria *Streptococcus pneumoniae* (major cause for pneumonia; Speert, 2006) were detected in the bacterial clean room community [Figure 21].

Though the ***Actinobacteria*** are only the third strongest phyla in this clean room, the *Actinobacteria* class represents more than 18.75% of all the collected uncultivable clean room bacteria making it, in fact, the second biggest class detected. Again the identified species are mainly associated with the two major contamination sources: the human flora (Gao *et al.*, 2007) and natural environments (Davis *et al.*, 2005). Once more, several of the identified species are opportunistic human pathogens like *Corynebacterium kroppenstedtii*, *Actinomyces odontolyticus* or *Micrococcus lylae*, which are recognized for their ability to cause actinomycosis (Cone *et al.*, 2003) or endocarditis (Kong *et al.*, 1998).

Another *Actinobacter* species (99.5% similarity to *Rothia aeria*) collected from outside the clean room (Hydra facility 3) was first described in 2004 by Ying Li and colleagues, who isolated the bacteria from the air collected inside the Russian space station MIR. This species is a good demonstration of the ability of bacteria to:

a) survive the unfavourable conditions of man-made controlled environments
b) reach highly controlled spaces (MIR-space station)
c) adapt and grow under these new conditions

This fact stresses the importance of continually monitoring the microbial community present in clean rooms to prevent the emergence and spreading of possible pathogenic or resistant bacteria which could cause significant health problems for staff working in clean rooms or astronauts living inside the international space station.

The gram-negative ***Proteobacteria*** are the second diverse and the most balanced of the mayor phyla observed in the tested clean room. The three genera of α-, β-, and γ-*Proteobacteria* represent respectively, 11.25%, 6.25% and 12.5% of the uncultivable bacterial community. The two missing

genera, the ε- and δ-*Proteobacteria,* were also found in this study but only within the clone fraction possessing a sequence similarity below the species identification threshold of 97%. In this study *Rickettsia honei*, a major human pathogen (cause of the spotted fever) of the *Alphaproteobacteria* class, was identified inside the clean room (Hydra facility 06).

Even though the used culture independent methods do not allow the quantitative analysis of the detected bacterial species, the huge variety and species diversity of the three mayor identified phyla emphasizes the dominance of these groups within the clean room community.

4.3.3 Analysis of the uncultivable bacterial species distribution inside the Hydra facility

The species distribution of the 80 detected bacteria within these three areas was analyzed next. It was noticeable that the vast majority of species (81%) were only found once or within one region of the facility. Still 15% of identified microorganisms were detected within two of the three analysed areas. Within this group of bacteria, correlating incidences were mostly detected between the out- and inside of the clean room. Judging from the bioburden and species distribution, the imposed additional requirements to enter the ATV were able to lower the bacterial presence inside the ATV. However, contamination could not be significantly reduced as the air flow between the clean room and the inside of the ATV was not controlled.

Only four species (4%) of the identified bacteria could be verified within all three regions. Three of these bacteria (*Streptococcus mitis, Streptococcus pneumoniae* and *Granulicatella adiacens*) are part of the normal human flora, whereas, the bacteria *Mycobacterium chitae* is a common isolate from environmental samples.

Streptococcus mitis is present in over 60% of the tested samples, rendering the bacterium, in fact, the most commonly detected uncultivable species within the ESA's Hydra facility.
Streptococcus mitis is involved in human health problems by causing endocarditis (Hall and Baddour, 2002) or serving as a reservoir for antibiotic resistance genes (Dowson *et al.*, 1993). Several publications document the fact that resistance factors identified in *Streptococcus mitis* were transmitted to the closely related human pathogen ***Streptococcus pneumoniae*** by horizontal gene transfer (Delorme *et al.*, 2007; Dowson *et al.*, 1997). In the last years the genome of the wild type and antibiotic resistant phenotype B6 of *Streptococcus mitis* were fully sequenced to study this process in detail (Hakenbeck *et al.*, 1998). Both *Streptococcus* species (*S. mitis* and *S. pneumoniae*) were identified in this study in the majority of tested locations. *Streptococcus mitis* was detected in 61% (11/18) and *Streptococcus pneumoniae* 22% (4/18) of all samples. This is an interesting correlation as both bacteria were always detected within the same samples (4/4). This proximity provides both species

with an opportunity to exchange genetic material (resistances). Furthermore, the fact that these species were not detected by culture-dependent methods confirms the necessity of direct DNA analysis in surveys of bacterial communities.

Once before *Streptococcus mitis* was the focus of the scientific interest when NASA scientists isolated a living specimen from a camera which was stationed for over two years on earth's moon (1967-69). Initially, this incidence fostered the theory that the bacterium could withstand unprotected the harmful effects of long term space travel. Today this claim cannot be sustained, and the option that the contamination of the hardware occurred after its recovery is favored (Glavin *et al.*, 2004).

Granulicatella adiacens was present in 33% of all tested areas. The commensal bacterium is part of the mucosal membranes of humans and can act as an opportunistic pathogen (endovascular infections; Senn *et al.*, 2006). The species was only detected by cultivation independent methods, which is not surprising as members of the *Granulicatella* family can often only be grown in the laboratory on specialised media or in co-culture with other bacteria (e.g. *Staphylococcus aureus*) (Ruoff, 2002).

Mycobacteria are known for their unique cell wall composure which grants the family a strong resistance against acids, alkalis, detergents or antibiotics (Russell, 1996). ***Mycobacterium chitae*** was one of the main species detected in the Hydra facility and present in 44% of all the sampled areas. Though this bacterium could not be detected using culture depend methods, the number of occurrences inside the controlled area and its strong cell wall composition makes it very probable that this species is capable of persisting under clean room conditions. An adaptation of the culturing conditions to the specific needs of the bacterium would certainly lead to cultivable isolates of *Mycobacterium chitae* from within ESA's clean room.

4.4 Phylogenetic comparison between the cultivable and uncultivable bacterial fraction

Using molecular- and microbial-based phylogenetic analysis methods side by side it was possible to identify 80 uncultivable and 18 cultivable bacterial species from ESA's clean room. Comparing the detected cultivation dependent and independent microbial diversity several key differences become apparent.

Examining the detected clean room population by direct DNA analysis almost 40% of the identified species belong to the gram-negative bacteria. But once the samples were cultivated the majority isolates (89%) were gram-positive bacteria. There are several possible explanations for this observed difference in the bacterial composition.

The majority of the detected uncultured gram-negative bacteria could be in the "viable but non-culturable" (VBNC) state. In this physiological condition the cells still exhibit metabolic signs of activity, but their ability to grow under the appropriate conditions (medium, temperature ect.) is lost (Lázaro *et al.*, 1999; McDougald *et al.*, 1998; Paludan-Muller, 1996). This state is triggered by species specific stress factors like starvation, visible light and temperature (Lázaro *et al.*, 1999; Oliver, 1993) which are all present in a clean room. Moissl and colleagues (2007) were able to detect the presence of bacteria in VBNC state in such environments supporting this possibility.

Also the majority of gram-negative bacteria could be lost during the sample handling processes [2.3.4.1]. These treatments (sonication and vortexing) could lead to the disruption and loss of the more fragile gram-negative bacteria. Though no influence from the two treatments could be detected during our experimental setup [3.1.1], the tests were done with spores or vegetative cells of *Bacillus subtilis,* a gram-positive bacterium. Therefore, these results can only be used as survival indicator for the gram-positive fraction and further tests with gram-negative bacteria need to be conducted to clarify this matter.

Assessing the most likely sources of the identified bacteria, a second profound difference is noticeable. Analysing the bacterial diversity using molecular methods identifies the flora of the human work crew as the dominant source for contamination. Every second uncultivated bacteria discovered (54%) is affiliated with this source. Once a cultivation step is included into the experimental setup, this percentage drops to 39%, leaving environmental habitats (soil, water) the main source (56%) of the contaminating bacteria [Table 15].

The difference in the composition between the cultivated and uncultivated bacterial species could arise by the fact that human associated bacteria are not as flexible in adapting to the changed and harsh conditions present in clean rooms as their environmental counterparts. Investigations of the Apollo and Viking space craft (Puleo *et al.*, 1973; Puleo *et al.*, 1977) and industrial or hospital clean rooms (Favero *et al.*, 1966; Favero *et al.*, 1968) identified the amount of human associated bacteria within the cultivable fraction of a clean room of up to 75%, rendering this possibility unlikely.

The second, and more likely, explanation for the change in the detectable community is based on the composition of the cultivation conditions applied in this study. Both media (R2A & TSB) used during the cultivation phase were created to grow environmental bacteria (Reasoner and Geldreich, 1985). For the cultivation of human associated bacteria several, more specialized media, e.g. blood or chocolate agar, are in use today (Ryan and Ray, 2004); and if applied, would certainly raise the number of cultivable human associated bacteria.

Overall when analyzing the species diversity identified by cultivation dependent and independent methods, it is striking that only a minor fraction (7%) of the cultured species were identified by both approaches. The fact that most of the cultivated bacteria are gram-positive fraction and only

marginally detected using direct DNA extraction could suggest an insufficient cell lysis step in the experimental setup (von Wintzingerode *et al.*, 1997). Using cultivation independent methods almost 60% of the identified species belonged to the gram-positive, a fact that argues strongly against an inadequate cell lysis step during sample processing.

The collected results rather propose that the dominant species detected by cultivation based techniques mostly represent minority species of the actual present bacterial community. This conclusion is supported by numerous publications which compare the diversity of cultivable and uncultivable bacterial communities (Kirk *et al.*, 2004; La Duc *et al.*, 2004; Moissl *et al.*, 2007; Tamaki *et al.*, 2005). These findings emphasize the fact that combinations of culture dependent and independent methods need to be applied to establish a (more) genuine description of any environmental bacterial community.

4.5 Resistance characteristics of the collected cultivable bacterial strains

Once the bacterial community of the clean room was phylogenetically identified by cultivation dependent and independent methods, the next objective of this study was to characterize the resistance capabilities of the collected isolates. A growing number of papers describe the variety of metabolic characteristics between different strains of the same species (Myers *et al.*, 2006; Saunders *et al.*, 2005). The field of metagenomics is addressing this very question by analyzing the genetic potential inherent in bacterial communities (Riesenfeld *et al.*, 2004; Steele and Streit, 2005). Through the extensive use of clone libraries and DNA sequencing scientist try to identify genes associated with different metabolic activities and enzymatic capabilities in the gene pools of numerous environments (Elend *et al.*, 2006; Gill *et al.*, 2006; Kennedy *et al.*, 2007; Schmeisser *et al.*, 2003; Streit and Schmitz, 2004). Applying this approach to this environment would lead to new information about the community's metabolic potential, in effect, opening new insights into the selection and adaptation processes occurring inside this man-made extreme environment.

Clean rooms represent an extremely selective environment which exposes the microorganisms present to highly restrictive conditions, e.g. low humidity, few carbon sources and possible application of biocidal agents (La Duc *et al.*, 2007). Consequently, it is likely that clean rooms are exerting a form of selective pressure on the present bacterial community favouring sturdy, highly resistant strains. To investigate if an observable raise in the resistance characteristics of the microorganisms present in a clean room could be detected, the collected cultivable bacterial isolates were subjected to a series of harmful treatments.

It is important to screen, if possible, all the individual strains of each species to ensure a correct overview of the resistance potential of the collected isolates due to three reasons:

I) The bacterial community is not uniformly distributed throughout the clean room but rather present in localized colonies
II) Interaction (spreading of resistances) between colonies is highly unlikely
III) Most of the detected bacteria will be carried in by the human workforce, making it highly likely that individual strains of one species will derive from different locations and origins

Therefore, gathered results can only represent the resistance behaviour of this specific bacterial strain and should not be applied to the bacterial species as a whole. In this study the response of over sixty isolated strains to more than twenty different treatments was tested. Once the workload became defined the need for a practicable, reliable resistance screening test became evident.

In a rapid and flexible test, the growth of the isolates was monitored spectrophotometrically as an increase in the optical density at 490 nm. Furthermore, by incorporating an unrestricted growth control (maximal growth) into the well setup, the gathered data could be normalised within each 96 well plate and compared to the resistance results from other isolates.

In this study the response of the 62 isolates to 23 inhibiting agents and conditions was tested. The treatments can be grouped into four distinct categories: antibiotics, heavy metals, biocides and environmental factors.

4.5.1 Antibiotics

Given that a large fraction of the collected bacteria is associated with the human normal biota, a steady exchange between the workforce's commensal flora and the clean rooms bacterial community can be expected. Thus, if a clean room in fact selects for sturdy and (antibiotic) resistant bacteria, it has to be assumed that these bacteria will be able to "reinhabit" the workers and thereby, be carried outside the restricted area again. Consequently, clean rooms could become a pool for the selection of resistant bacteria (Lancaster *et al.*, 2003). The fact that antibiotics selectively target prokaryotic organisms is the reason that antibiotics are the major therapeutic tool for the treatment of bacterial infections (Discotto *et al.*, 2001). As mentioned before, a growing number of bacterial strains exhibit resistance towards several different antibiotics and the human health problem that is created by this fact is well documented (WHO 2001; Struelens *et al.*, 2004). Especially in clean rooms used for the assembling of hardware for human based space mission, a raise in the antibiotic resistance of the present bacteria could pose a severe problem. Several studies established the fact that the human immune system weakens during space flight missions (Shearer, 2001; Sonnenfeld *et*

al., 2003) making humans in space especially susceptible to (bacterial) infections. If, these micro-organisms additionally developed a resistance to standard medication, minor infections could become a serious hazard (Klaus *et al.*, 2006).

To test the antibiotic resistance potential of the isolates the bacterial strains were subjected to six different classes of antibiotics which inhibit microbial growth by dissimilar mechanism.

When observing the effect the different antibiotics exert on the tested bacterial isolates, two distinguishable community-wide reactions can be observed. For antibiotics like penicillin, more than half of the tested bacteria did not exhibit any growth inhibition even in the presence of the highest applied concentration (100 µg/ml) [3.6.2]. A strong resilience to penicillin is not uncommon in other environmental samples where antibiotic resistance was developed as a result of bacterial exposure to antibiotics used in farming (Ash *et al.*, 2002; Burgos *et al.*, 2005). On the other hand, gramicidin (100µg/ml) was able to inhibit bacterial growth in almost all of the tested isolates [3.6.2]. The efficiency of Gramicidin against the bacteria isolated from inside the Hydra facility can be explained by the fact that all the tested cultivable isolates mainly belong to the group of gram-positive bacteria against which this ionophore is especially effective (Kondejewski *et al.*, 1996)

It was further noticeable that bacterial strains associated with the human flora (*Staphylococcaceae* family) exhibited a higher variance in their individual resistance potentials than isolates deriving from soil or environmental habitats (*Bacillaceae* family). It is probable that environmental organisms associated with dust or soil particles are carried, less frequently, but in larger aggregates into the clean rooms and are then distributed throughout the facility. Whereas human associated bacteria are continuously shed in minor amounts into the clean room by the workforce and, therefore, derive from several different individual habitats (humans). A number of studies were able to demon-strate the human flora is highly individual (Brown *et al.*, 2007; Gao *et al.*, 2007) and resistance characteristics can be strongly influenced by previous antibiotic treatments or other individual incidents (Reinthaler *et al.*, 2003). This theory could explain the more evenly occurrence resistance characteristics of soil based bacteria compared to the highly individual resistance potential of human associated strains. This is a significant observation which deserves further investigation.

One strain of *Bacillus subtilis* (EU071564) was noticed by its unusual antibiotic resistance profile. While bacteria of the *Bacillus subtilis* species are known to produce polypeptide antibiotics (e.g. polymyxin, difficidin, subtilin, mycobacillin) and therefore are immune against their specific harmful effects (Cao and Helmann, 2002), other antibiotics are known to suppress this species quiet effectively. For most antibiotics the minimal inhibitory concentrations (MIC) lie within the range of 0.1 to 16 µg/ml (Citron and Appleman, 2006; Dautle *et al.*, 2004). Wherefore the ability of this

specific strain to exhibit partial growth under the influence of up to 100 µg/ml of the respective antibiotic is remarkable. The isolate was only moderately influenced by the presence of ciprofloxacin (39%), trimetrophin (47%) and nisin (45%) and even grew almost unimpeded in the presence of gramicidin (80%). Nonetheless the strain showed a decrease in the ability to survive in the presence penicillin where only 40% of the unhindered, maximal growth could be detected. As two of the antibiotics, penicillin and gramicidin, affect the bacterial cell wall, it is possible that this strain gained part of its resistance by a change in the composition of its outer layer. As a more tightly cross-linked cell wall could probably help withstanding the damaging effect of gramicidin-created pores the extensive cross-linking would be more susceptible to the effect of penicillin which directly targets this process (Rice, 2006).

4.5.2 Heavy metals

Metals play an integral role in the functionality of the microbial cell. Several, so-called essential metals, like cobalt, copper, iron, ect. are utilized by the cell for redox-processes, protein stabilization or are crucial components of cellular enzymes (Bruins *et al.*, 2000). Other non-essential metals (e.g. silver, aluminium, cadmium, gold, lead and mercury), have no known cellular role and can be toxic for bacterial cells (Nies, 1999). While some of these metals (e.g. Zn, Fe and Cu) are, in low amounts, needed essentials and act toxic at higher concentrations (Hare, 1992) others, like mercury and cadmium are known to inhibit the enzymatic activity of metabolic process already at minute concentrations (Madigan *et al.*, 2003)

Presently six metal resistance mechanisms are postulated to exist:
1) exclusion by permeability barriers, 2) intra- and 3) extra-cellular sequestration, 4) active transport efflux pumps, 5) enzymatic detoxification and 6) reduction in sensitivity of cellular targets to metal ions (Bruins *et al.* 2000). Though genes encoding resistance systems can be found on bacterial chromosomes (e.g. arsenic, mercury and cadmium resistance in *Bacillus*) they are frequently located on plasmids which can be passed between bacterial species of one community (Silver, 1998). In recent years several authors recognized that some of the heavy metal resistance mechanisms do also protect the bacterial cells against the lethal effect of antibiotic (Ugur and Ceylan, 2003; De Ramaiah, 2007).
In this study the effect of six different heavy metals, ranging from slightly toxic like copper and zinc to highly damaging like mercury and cadmium on the cultivable bacterial populace was surveyed.

While most of the tested heavy metals affected the bacterial isolates quite strongly, single individual strains were identified which exhibited an unusually raised resistance potential against single heavy metals effects [3.6.3]. Once more a strain of *Bacillus subtilis* (EU071564), already mentioned in [4.5.1]

for its noticeable antibiotic resistance, was one of the few tested strains able to grow under the damaging influence of several tested heavy metals. The occurrence of heavy metal as well as antibiotic resistance, as it is evident in this strain, could be based on the fact that several resistance mechanisms can protect bacteria against the harmful effect of heavy metals as well as antibiotics (Baker-Austin *et al.*, 2006). If this broad resistance capability is caused by a few multiple resistance mechanisms or, for example, by the transformation of the bacteria with a plasmid containing multiple resistance determents (Paterson, 2006) needs to be investigated further.

A strain of *Staphylococcus haemolyticus* (EU071615) was able to survive in the presence of most all the tested heavy metals. The strain showed no (100% growth) or only minor inhibition (72% growth) while influenced by arsenic (100 mM) or zinc (100 mM), respectively. While being exposed to copper (100 mM) and cobalt (100 mM) the bacterium was still able to exhibit minor growth (27% and 27%). Only the exposure to mercury (10 mM) and cadmium (100 mM) kept the growth of this strain below 20% of the unrestricted maximum. Though several of the tested strains were able to survive better under single heavy metal treatments [3.6.3], this strain was remarkable in its ability to resist the inhibiting effect from four of the six tested metals. The species *Staphylococcus haemolyticus* is known to exhibit highly antibiotic resistant phenotypes. Once the whole genome was sequenced in 2005, several open reading frames were discovered which are known to confer heavy metal resistance (Takeuchi *et al.*, 2005). Oger and colleagues (2003) were able to identify a cadmium resistance (*cadA*) gene present in staphylococcal bacteria isolated from contaminated estuary in France. This specific cadmium resistance characteristic was missing from the isolate in our study, but it was resistant to a range of other heavy metals tested.

While it is highly unlikely that the bacteria were exposed to heavy metals inside the clean room, it is probable that the strains either posses a natural resistance (Nies, 1999) or that they acquired the resistance before they were brought into the clean room. Moreover, part of the identified heavy metal resistances can be cross resistance deriving from an adaptation of the bacteria to another influence (e.g. antibiotics treatment) (Baker-Austin *et al.*, 2006; Dhakephalkar *et al.*, 1994). Therefore, it is likely not a directly developed resistance against the harmful effect of the heavy metals but rather an indirect acquisition of the bacteria enabling it to withstand this lethal effect. The fact that several publications were able to demonstrate that resistances can be maintained within a bacterial strain, even in the absence of a selection pressure (Enne *et al.*, 2004; Gillespie, 2001), supports the possibility that the actual acquisition of the detected resistance occurred outside the facility.

4.5.3 Biocides

Chemical and physical antimicrobial agents are extensively used for sterilization of medical care units, industrial clean rooms or for the preservation of foods, pharmaceuticals and cosmetics (Lenczewski and Kananen, 1998; Reichert and Schultz, 1997 and Salvat *et al.*, 1995). Biocides are an integrated and important part of the infection control practices inside hospitals to prevent nosocomial infections (Rutala, 1995).

The collected cultivable isolates were exposed to six different biocides belonging to four distinct anti-microbial groups to determine the biocide resistance potential of the collected cultivable bacteria.

Alcohols, like the tested ethanol and isopropyl, are lethal for vegetative cells but exhibit no sporicidal activity (Knight and Cooke, 2002). The main inhibitory mechanism of alcohols is their protein denaturing effect though secondary effects, the inhibition of DNA, RNA, protein and peptidoglycan synthesis, have been observed in *E.coli* cells (Maillard, 2002). Both applied alcohols were able to severely inhibit all the tested isolates, in effect limiting the growth of most strains below the detection limit.

The second group, the **aldehydes**, were represented by glutaraldehyde and formaldehyde which both are highly bactericidal and sporicidal. Likely inhibitory targets of this group are cellular bio-molecules (e.g., proteins, RNA, DNA) and the cell wall (Denyer and Stewart, 1998). Once more all the tested isolates were severely inhibited in their growth, the only exception is a *Bacillus subtilis* strain isolated from outside the clean room (EU071564) which was able to exhibit minor growth under the influence of glutaraldehyde. It is known that *Bacillus subtilis* spores can withstand the damaging effect of the biocide to some extent (Russell, 1990); but as the aldehyde was added to the media and constantly present during bacterial growth, it would have affected the vegetative cells of the strains. Up to now, the resistance mechanisms of vegetative cells to glutaraldehyde have been poorly characterized. In 1999, Manzoor and colleagues isolated two resistant mutants exhibiting an increase in surface hydrophobicity and changes in the monosaccharides of the arabinogalactan and arabinomannan fractions of their cell wall. Though they could not fully identify the resistance mechanism, they postulate that these changes decrease the permeability of the cell wall towards aldehydes, and thereby, preventing the reagent to enter the cell and interact with the proteins.

Hydrogen peroxide generates radicals which can affect vegetative cells as well as spores. The antimicrobial effect of hydrogen peroxide is due to the generation of hydroxyl radicals which damage biological material (Denyer and Steward, 1998). The bacterial enzyme catalase, a natural defence against oxidative damage, cancels the harmful effect of the oxidant by decomposing the reagent into its non-hazardous elements: water and oxygen. As a number of the tested bacterial species are

known to produce catalyse, it is not surprising that this treatment was the least effective biocide tested (Madigan *et al.*, 2003).

UV-irradiation was chosen as representative for physical biocides due to the fact that it is widely applied for large surface disinfection as it is used in clean rooms (La Duc *et al.*, 2007). The damaging effect of UV is mainly through DNA base modification (Moeller et al, 2007), though at high UV fluency single and double strand breaks are documented (Slieman and Nicholson, 2000). Applying a UV-C dose of 25 J/m^2 will in average affect and alter 9 x 10^3 bases of any unprotected vegetative *Bacillus subtilis* cell (Moeller, unpublished data). Healthy bacterial cells are able to repair this kind of damage to their DNA in the low dosage range by utilizing their natural DNA repair mechanisms (Sinha and Hader, 2002). Yet at higher doses of 750 J/m^2 and above, the cells standard repair mechanisms are overtaxed and cannot compensate the damage done to the DNA. Only a few highly resistant bacteria like *Deinococcus radiodurans*, or protected cell forms like spores are known to survive such doses (Kitayama *et al.*, 1983). During the screening the isolates were subjected to several different doses of UV-C irradiation ranging from low dosage (100 J/m^2) to highly lethal (2000 J/m^2) [2.4.7]. Whereas most isolates were able to compensate the damaging effect of low dose (100 J/m^2) UV-C irradiation, no strain was able to grow after irradiation with UV-C in the high dosage range (1000-2000 J/m^2).

Overall every tested biocide was highly effective against the isolates, a result not encountered with any of the other three categories. A possible explanation for this sensitivity could lie within the species composition of the cultivable community. Morton and colleges (1998) were able to demonstrate that gram-positive bacteria are more susceptible to the damaging effect of biocides as a result of their weaker outer membrane structure (Madigan *et al.*, 2003). As all of the isolated species are gram-positive, this could be an explanation for the strong inhibitory effect of the tested biocides on the cultivable clean room community.

4.5.4 Environmental conditions

The final group of tested conditions is composed of five treatments which reflect natural occurring severe conditions. The isolates were tested for their desiccation tolerance (seven days), halotolerance (1M NaCl), acidotolerance (pH 4), alkalotolerance (ph 9) and their ability to withstand a heat shock (80°C, 10 min). While most of these conditions can be easily survived by most *Bacillaceae* in spore-form (Claus and Berkeley, 1986), the experimental setup used vegetative cells and the pH shifts and high salt concentration were constantly present during the bacterial growth phase to circumvent false positive results due to spore formation [2.4].

Analyzing the resistance characteristics of the individual isolates, it was noticeable that most of the strains able to grow under the tested conditions belong to the *Bacillaceae* family [3.6.5]. This phenotypically and genotypically heterogeneous family is mainly associated with environmental habitats (Claus and Berkeley, 1986) which can vary strongly in composition and during seasonal changes (Priest, 1993). Therefore, most of these bacteria are physiologically flexible and able to respond quickly to alterations in the e.g. pH, salinity, temperature etc. which are all conditions tested in this category. This adaptability is well documented for members of the *Bacillaceae* family which are globally distributed and able to grow under a broad range of different and changing conditions (Priest, 1993).

A *Bacillus atrophaeus* strain cultivated from outside the restricted area (EU071547) exhibited a remarkable ability to grow almost unhindered at low (92%) and high pH (97%). While the strain also demonstrated a tolerance to desiccation (65%), it was not able to grow (0%) in the presence of 1M NaCl. It was overall evident that isolates of the *Bacillus* species were more susceptible (ca. 15-20% growth) to the high saline condition, whereas strains of the *Staphylococcaceae* family were able to tolerate the presence of 1M NaCl (ca. 50% growth). In literature *Staphylococcaceae* are described as halotolerant (Wydro *et al.*, 1977). The microbes feature effective ion pumps which enable the bacteria to sustain a low (physiological) intracellular ion concentration even in the presence of high amounts of extracellular salt (up to 15%).
Analyzing the pH of the medium from isolates exhibiting growth under the tested alkaline or acidic conditions, two distinct survival strategies were detected. Several isolates were able to modulate the pH of the medium towards the neutral range, in effect adjusting the severe growing conditions towards their needed optimum (Kumar and Takagi, 1999). Other isolates tolerated the pH shifts and were able to grow under the tested circumstances.

As mentioned, most of the human associated bacteria exhibited a rather low tolerance for the tested stresses. Nevertheless, compared to other members of this species, a strain of *Staphylococcus epidermidis* collected from inside the clean room (EU071604) displayed an elevated ability to grow under the tested conditions. While the strain was able to moderately grow at pH 4 and pH 9 (42% and 34%), it was capable of tolerating desiccation and high salt concentration conditions (52% and 73%). As discussed above member of the *Staphylococcus* are known to be halotolerant, but most strains were not able to exhibit any growth under changed pH conditions. A more in depth characterization of this strain could provide insights into new, undescribed resistance mechanisms.

4.5.5 Resistance comparison between the strains of one species

As reasoned in [4.5], it is likely that the origin and, thereby the capabilities of the collected clean room isolates can vary quiet strongly between different strains of the same species. The resistances investigation in this study was, therefore, developed to be able to test all the individual isolates [2.4]. This setup made it possible to analyze multiple strains, collected from different areas inside the facility, for most of the identified species.

The screening of strain specific resistance is standard procedure for clinical antibiotic resistance testing (Rodrigues da Silva *et al.*, 2004) and, in recent years, has become more common with environmental samples (Branco *et al.*, 2005). This detailed, strain specific, resistance testing is not necessarily needed in, for example, enclosed liquid environments where bacteria are in constant motion and can easily transfer genetic material (Wright, 2007). It is vital for a realistic survey of clean room environments, where the diversity of interactions of the highly localized heterogeneous bacterial community will only take place in separate locations/colony/biofilms.

The data gathered in this study [3.6.6] supports the importance of strain specific resistance testing. Comparing the responses of individual strains of one bacterial species to the harmful effects, it was noticeable that several of the strains exhibited variations compared to the "average" collected or literature described species resistance characteristics. As noticed with a strain of *Staphylococcus epidermis* [Figure 26A] gathered from inside the controlled area (EU071608), a strong resistance to the antibiotic rifampin was noticed. The strain was able to grow uninhibited (100%) in the presence of rifampin (10 µg/ml), whereas other strains of the collected specie were severely inhibited in their growth (max 40% of untreated growth). It is known that rifampin resistance can be the result of point mutations in the *rpoB* gene (Jin and Gross, 1988; Kapur *et al.*, 1994). This mutation will lead to an amino acid exchange in the β-subunit of the RNA polymerase leaving the enzyme functional but effectively blocking the binding and thereby inhibition by rifampin. Therefore, it is probable that a point mutation occurred in this individual strain leading to this strain's rifampin resistance. Though to confirm this theory, the *rpoB* gene of the strain needs to be sequenced.

These variations are not only seen with the tested antibiotics but were observable throughout the different resistance categories. Another example is the mercury resistance of a *Bacillus subtilis* strain (EU071572) [Figure 26B]. The isolate exhibits an unimpeded growth (100%) under the influence of mercury (10 mM). This resilience could have been acquired by the uptake of a plasmid containing the *mer* gene operon. This operon confers the ability to transport the toxic Hg^{2+} ions across the cell membrane into the cell where it is detoxified by another operon encoded (*merA*) enzyme: the mercuric reductase (Silver, 1998). Tothova and colleagues (2006) observed the transfer of a *merA* encoding plasmids from mercury-resistant soil bacteria to a *Bacillus subtilis* strain isolated from the

rumen of a cow. This study represents a good example for the common exchange of genetic material between bacteria from different habitats, explaining the emergence and spreading of resistances within in the bacterial collective and over local or environmental “boundaries”.

Even though only the increase of the resistance characteristics was discussed, it has to be mentioned that both variations, a heightened sensitivity as well as an increased resistance, were observed in individual strains.

Summarizing, several of the collected and tested bacterial strains exhibited the ability to tolerate the harmful conditions applied in this study. While a number of strains were able to resist one or two of the exerted stresses, only a few bacteria were able to tolerate multiple harmful conditions. Some of these identified resistances can be attributed to species specific features but a number of isolates exhibited species uncharacteristic resistances capabilities. It is highly likely that these abilities derive from changes in the strains genotype and represent individual, strain specific, resistances.
To characterize and identify the detected resistance further, more in-depth tests need to be conducted to discover and decode the molecular basis underlying this resistance.

4.6 Outlook

The results compiled during this study emphasize the necessity to modernize the standard hardware contamination screening protocols applied by ESA. The high bacterial diversity, which could only be detected by cultivation independent techniques, strongly supports the rational of integrating DNA extraction and analysis methods into the survey setup. Therefore, to be able to investigate and study the actual bioburden of a space craft, molecular based methods need to be incorporated into ESA's planetary protection protocols.

As this study was able to provide more detailed information about the bacterial community of the Hydra facility, further direct DNA extraction and 16S rDNA studies should be conducted to complete the survey. It should also be mentioned, that the samples were only obtained during one season. The tracking of the facility's bacterial composition over several years or missions, could lead to new insights about the bacterial community formation process inside a clean room environment.

The bacterial diversity of the uncultivable community was only qualitatively identified during this study and further investigations should be conducted to verify the specific quantity of the different detected bacterial species. As a result of this study, species specific, molecular probes can now be utilized to identify and quantify the bacterial distribution (real time PCR) inside the facility.

Now that protocols are established to extract genomic DNA from swabs used during clean room contamination control, a whole range of possible new analysis opportunities opened up.

I) The pathogenic potential of the bacterial community could be monitored for the emergence of pathogenic genes or virulence factors.

II) The genomic potential of the bacterial community could be tested and screened for useful biocatalysts (metagenomics).

III) The bacterial community's ability to damage sensitive electronics by i.e. corroding or degrading of the applied hardware needs to be surveyed to prevent possible breakdown due to bacterial corruption.

Furthermore, changes in the utilized clean room materials or of the air composition could be examined to further deprive the bacteria of nutrition and inhibit their growth.

Concerning the raised resistance exhibited by several bacterial strains, more in depth analysis of that characteristic needs to follow. By discovering the specific molecular mechanisms responsible for the noticed raised resistances it would be possible to identify the origin of the ability or even diagnose if a clean room environment exerts a selective effect on the bacteria. Concerning human space flight missions, especially the spreading of antibiotic resistance within the community needs to be addressed in more detail and monitored closely.

V. Thesis summary

This thesis is part of ESA's effort to advance Europe's employed planetary protection policy to avoid unwanted contamination of our solar bodies due to mankind's spaceflight program.
Consequently the biological contamination of each spacecraft is monitored and, if necessary, reduced before launch. Therefore, the goal of this thesis was to identify and analyse the contamination level and phylogenetic diversity present in a European class 100,000 clean room (no more than 100k particles larger than 5 µm per foot^3). In addition, the physiological properties of the cultivable bacteria collected from inside the facility were examined to identify possible changes in the bacterial characteristics due to the selective conditions present in such an environment. To achieve these goals three lines of work have been pursued:

I. Evaluation and optimization of the established microbial sampling protocols

Clean rooms represent a low biomass environment for which an effective and sensitive analysis of the collected samples is fundamental. The first step of this thesis was to critically evaluate existing microbial sampling protocols typically applied for the bioburden control of clean rooms. The swab technique, as specified by NASA (NPG: 5340.1D, 1980), was analysed by spiking an area with a defined number of spores and comparing the results of the swabbing method with the actual number of spores applied. During the analysis two major weaknesses in the sampling process were identified. First, only 50-60% of the actual spores present were picked up by the swab during sampling. Second, from those spores picked up, only 50% were again detachable from the swab and contribute to the final contamination count. These two factors will lead to an overall underestimation of the real bioburden by up to 300% (Nellen *et al.*, 2006). Once the analysis revealed that half of the collected microorganisms are retained in the swab, it was tested if this major shortcoming could be utilized. Several DNA extraction techniques were tested and combined to establish an effective method of directly isolating DNA from the swab-heads used during sampling. Specific protocols were developed, and it is now possible to identify cultivable and uncultivable bacteria from the same clean room sample. The results of this optimization work will be incorporated into Europe's future planetary protection bioburden monitoring protocols of space craft assembly facilities.

II. Identification of the cultivable and uncultivable bacterial phylogenetic diversity of a class 100k clean room facility

For this purpose ESA's hardware testing facility (class 100k clean room) at ESTEC, (Noordwijk, Netherlands) was chosen as model system for a test sampling. After the sample collection, aerobic cultivation techniques and 16S rDNA based relatedness comparison were used in combination to identify the bacterial phylogentic variety present inside the facility. This work represents the first phylogenetic study of the cultivable and uncultivable bacterial fraction inside a European clean room.

The integration of direct DNA analysis into the standard sampling assay raised the number of detected bacteria from 18 to 80 distinguishable species (≥ 97% 16S rDNA sequence similarity). Furthermore, it was possible to extract and identify the uncultivable bacterial fraction from each of the sampled locations; whereas, some of the locations did not yield any cultivable bacteria. This increase in phylogenetic data by molecular based methods is a known fact (Amann *et al.*, 1995) and emphasizes the importance of including such methods into ESA's bioburden monitoring process. Over 90% of the identified bacteria could be associated with three phyla, the *Firmicutes* (45%), the *Actinobacteria* (18.75%) and the *Proteobacteria* (30%). These 3 phyla are typical representatives of environmental and human associated samples and correspond to the two main contamination sources identified in this study: the human work crew (52%) and the urban surroundings outside the clean room (40%). Most of the detected bacterial species are harmless for the human work crew, nonetheless several pathogenic species were detected. These bacterial families should be monitored more closely during later studies. Only four species (*Granulicatella adiacens, Mycobacterium chitae, Streptococcus mitis* and *Streptococcus pneumoniae*) could be identified in each of the three sampled access-restriction classes of the ESTEC facility.

III. Analysis of the resistance potential of the collected cultivable bacteria

Additionally, the question was addressed if the highly restrictive clean room environment could lead to a change in the physiological properties of the present bacterial community. The hypothesis was tested by using a microtiter based screening procedure to analyse the resistance potential of the cultivable bacterial community. The resistance characteristics of 62 collected isolates was tested against a selection of 23 harmful treatments. Though the difference in potency between different treatments (mildly to highly damaging) could be detected on the community level, no raised community resistance was observable under the tested conditions. It was striking that a high variance in the resistance characteristics between individual strains of the same species could be observed.

Additionally, during this study several strains were identified which exhibit a marked increase in their resistance against one or more of the tested influences. These results lead to the conclusion that though changes in individual strains seemed to be promoted by the clean room conditions, no community level changes can be observed under the tested conditions. Furthermore, the molecular changes leading to the observed resistances need to be examined in detail to identify the source and if these resistances were obtained while the bacteria were inside the clean room or before they were brought into the facility.

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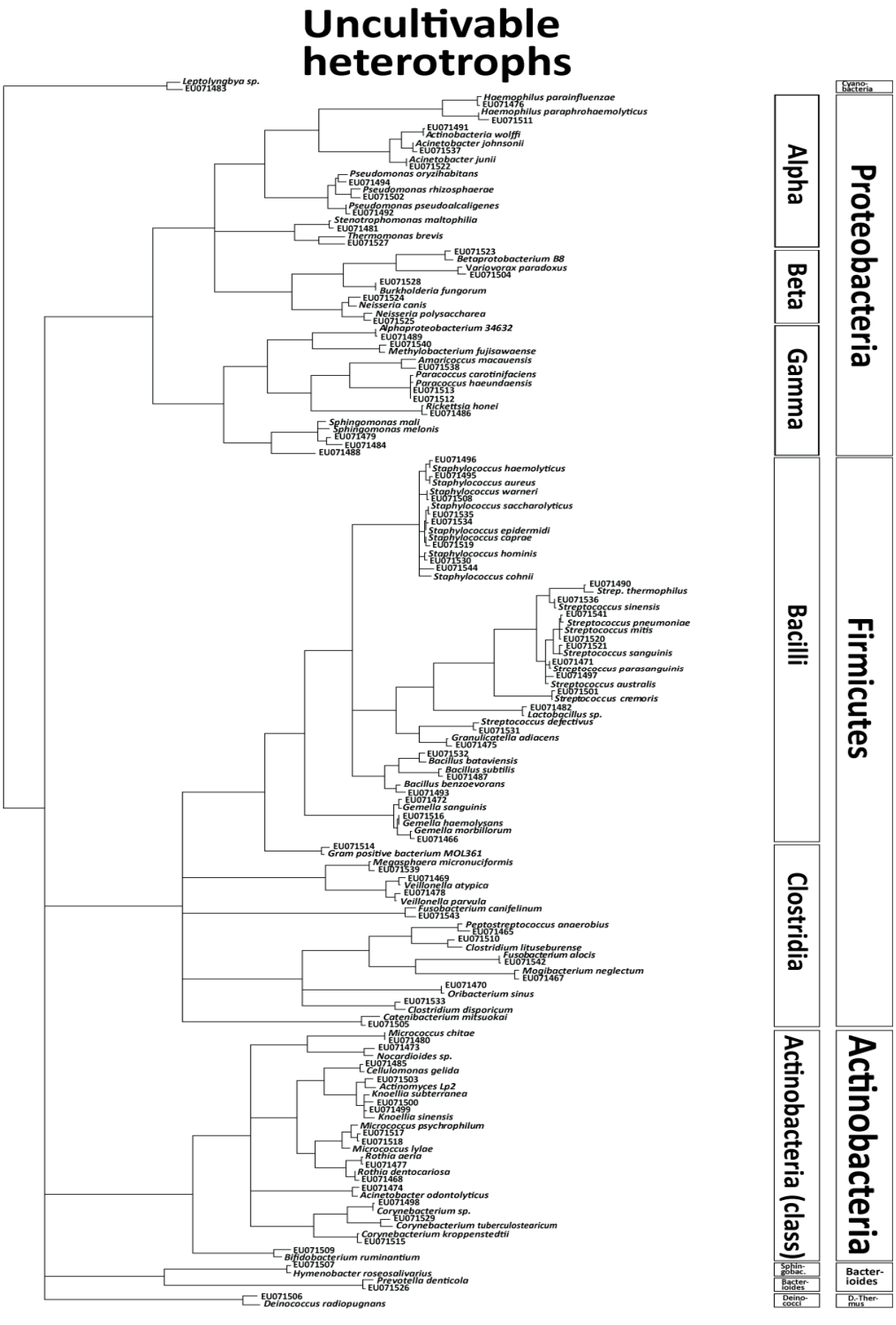

Figure 27: Phylogenetic tree of the identified uncultivable bacterial species.
This Figure is also stored on the Data-CD.

The uncultivable distance-matrix is only stored on the Data-CD as the Table is too large to be printed out in detail.

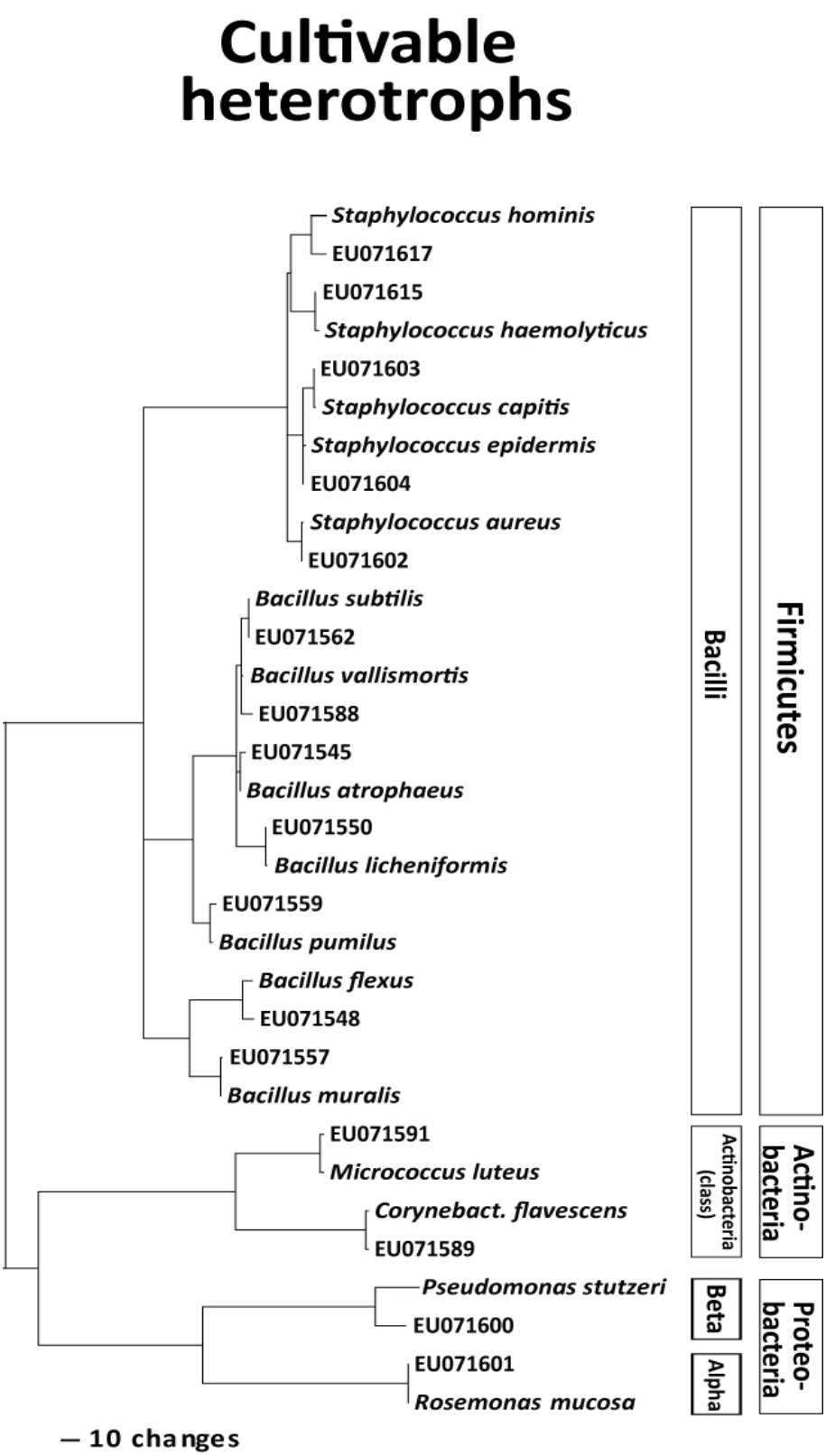

Figure 28: Phylogenetic tree of the identified cultivable heterotrophic bacterial strains.
This Figure is also stored on the Data-CD.

Table 17: Distance-matrix of the identified cultivable heterotrophic bacterial strains.
This Table is also stored on the Data-CD.

	Staphylococcus hominis	Bacillus subtilis DSM10	EU071591	EU071545	EU071603	Pseudomonas stutzeri 270039	EU071550	EU071562	Bacillus vallismortis	Staphylococcus aureus	Bacillus flexus	Staphylococcus epidermidis	EU071565	Corynebacterium flav 270036	EU071601	EU071602	Micrococcus luteus 270037	EU071559	EU071615	EU071548	EU071557	EU071617	EU071604	EU071588	Roseomonas mucosa 27003A	Bacillus licheniformis	Bacillus atrophaeus	Staphylococcus capitis	EU071589	Bacillus pumilus DSMZ27	Bacillus muralis 270030	Staphylococcus haemolyticus
Staphylococcus hominis	1,00																															
Bacillus subtilis DSM10	0,92	1,00																														
EU071591	0,81	0,82	1,00																													
EU071545	0,92	0,99	0,82	1,00																												
EU071603	0,98	0,92	0,81	0,92	1,00																											
Pseudomonas stutzeri 270039	0,79	0,79	0,78	0,79	0,79	1,00																										
EU071550	0,92	0,99	0,82	0,98	0,92	0,79	1,00																									
EU071562	0,92	1,00	0,82	0,99	0,92	0,79	0,99	1,00																								
Bacillus vallismortis	0,92	1,00	0,82	0,99	0,92	0,79	0,98	1,00	1,00																							
Staphylococcus aureus	0,98	0,92	0,81	0,92	0,99	0,78	0,92	0,92	0,92	1,00																						
Bacillus flexus	0,92	0,94	0,81	0,94	0,92	0,79	0,94	0,94	0,94	0,93	1,00																					
Staphylococcus epidermidis	0,99	0,92	0,81	0,92	0,99	0,79	0,92	0,92	0,92	0,99	0,92	1,00																				
EU071565	0,79	0,79	0,78	0,78	0,79	0,97	0,79	0,79	0,79	0,78	0,78	0,78	1,00																			
Corynebacterium flav 270036	0,78	0,79	0,90	0,79	0,78	0,77	0,79	0,79	0,79	0,78	0,79	0,78	0,78	1,00																		
EU071601	0,78	0,80	0,78	0,80	0,78	0,82	0,80	0,80	0,80	0,78	0,79	0,78	0,82	0,78	1,00																	
EU071602	0,98	0,92	0,81	0,92	0,99	0,78	0,92	0,92	0,92	1,00	0,93	0,99	0,78	0,78	0,78	1,00																
Micrococcus luteus 270037	0,81	0,82	1,00	0,82	0,81	0,78	0,82	0,82	0,82	0,81	0,81	0,81	0,78	0,90	0,79	0,81	1,00															
EU071559	0,92	0,97	0,82	0,97	0,92	0,79	0,96	0,97	0,97	0,93	0,94	0,93	0,79	0,80	0,80	0,93	0,82	1,00														
EU071615	0,99	0,92	0,81	0,92	0,98	0,79	0,92	0,92	0,92	0,99	0,92	0,99	0,79	0,79	0,78	0,99	0,81	0,92	1,00													
EU071548	0,92	0,94	0,82	0,93	0,92	0,79	0,94	0,94	0,94	0,92	0,99	0,92	0,79	0,79	0,79	0,92	0,81	0,95	0,92	1,00												
EU071557	0,91	0,93	0,82	0,93	0,92	0,80	0,94	0,93	0,93	0,92	0,96	0,92	0,80	0,80	0,79	0,92	0,82	0,94	0,92	0,96	1,00											
EU071617	1,00	0,92	0,81	0,92	0,98	0,79	0,92	0,92	0,92	0,98	0,92	0,99	0,79	0,78	0,78	0,98	0,81	0,92	0,99	0,92	0,91	1,00										
EU071604	0,98	0,92	0,81	0,92	1,00	0,79	0,92	0,92	0,92	0,99	0,92	1,00	0,79	0,78	0,79	0,80	0,92	0,82	0,97	0,92	0,93	0,92	0,98	1,00								
EU071588	0,92	0,99	0,82	0,99	0,91	0,79	0,98	0,99	0,99	0,92	0,93	0,91	0,78	0,79	0,80	0,92	0,78	0,79	0,80	0,78	0,79	0,78	0,78	0,91	1,00							
Roseomonas mucosa 27003A	0,78	0,80	0,78	0,80	0,78	0,82	0,80	0,80	0,80	0,78	0,79	0,78	0,82	0,78	1,00	0,78	0,79	0,80	0,78	0,79	0,79	0,78	0,78	0,80	1,00							
Bacillus licheniformis	0,92	0,99	0,82	0,98	0,92	0,79	1,00	0,99	0,98	0,92	0,94	0,92	0,79	0,79	0,80	0,92	0,82	0,96	0,92	0,94	0,94	0,92	0,92	0,98	0,80	1,00						
Bacillus atrophaeus	0,92	0,99	0,82	1,00	0,92	0,79	0,99	0,99	1,00	0,92	0,94	0,92	0,79	0,79	0,80	0,92	0,82	0,97	0,92	0,94	0,93	0,92	0,92	0,99	0,80	0,99	1,00					
Staphylococcus capitis	0,98	0,91	0,81	0,92	1,00	0,79	0,92	0,91	0,92	0,99	0,92	0,99	0,79	0,78	0,78	0,99	0,81	0,92	0,98	0,92	0,92	0,98	0,99	0,91	0,78	0,91	0,92	1,00				
EU071589	0,78	0,79	0,90	0,79	0,78	0,77	0,79	0,79	0,79	0,78	0,79	0,78	0,78	1,00	0,78	0,78	0,90	0,80	0,79	0,79	0,80	0,78	0,78	0,79	0,78	0,79	0,79	0,78	1,00			
Bacillus pumilus DSMZ27	0,92	0,97	0,82	0,97	0,93	0,79	0,96	0,97	0,97	0,93	0,95	0,93	0,79	0,80	0,80	0,93	0,82	1,00	0,93	0,95	0,94	0,92	0,93	0,97	0,80	0,96	0,97	0,92	0,80	1,00		
Bacillus muralis 270030	0,91	0,93	0,82	0,93	0,92	0,80	0,93	0,93	0,93	0,92	0,96	0,92	0,80	0,80	0,79	0,92	0,82	0,94	0,92	0,96	1,00	0,91	0,92	0,93	0,79	0,94	0,93	0,92	0,80	0,94	1,00	
Staphylococcus haemolyticus	0,99	0,92	0,81	0,92	0,98	0,79	0,92	0,92	0,92	0,99	0,92	0,99	0,79	0,79	0,78	0,99	0,81	0,92	1,00	0,92	0,91	0,99	0,99	0,92	0,78	0,92	0,92	0,98	0,79	0,92	0,91	1,00

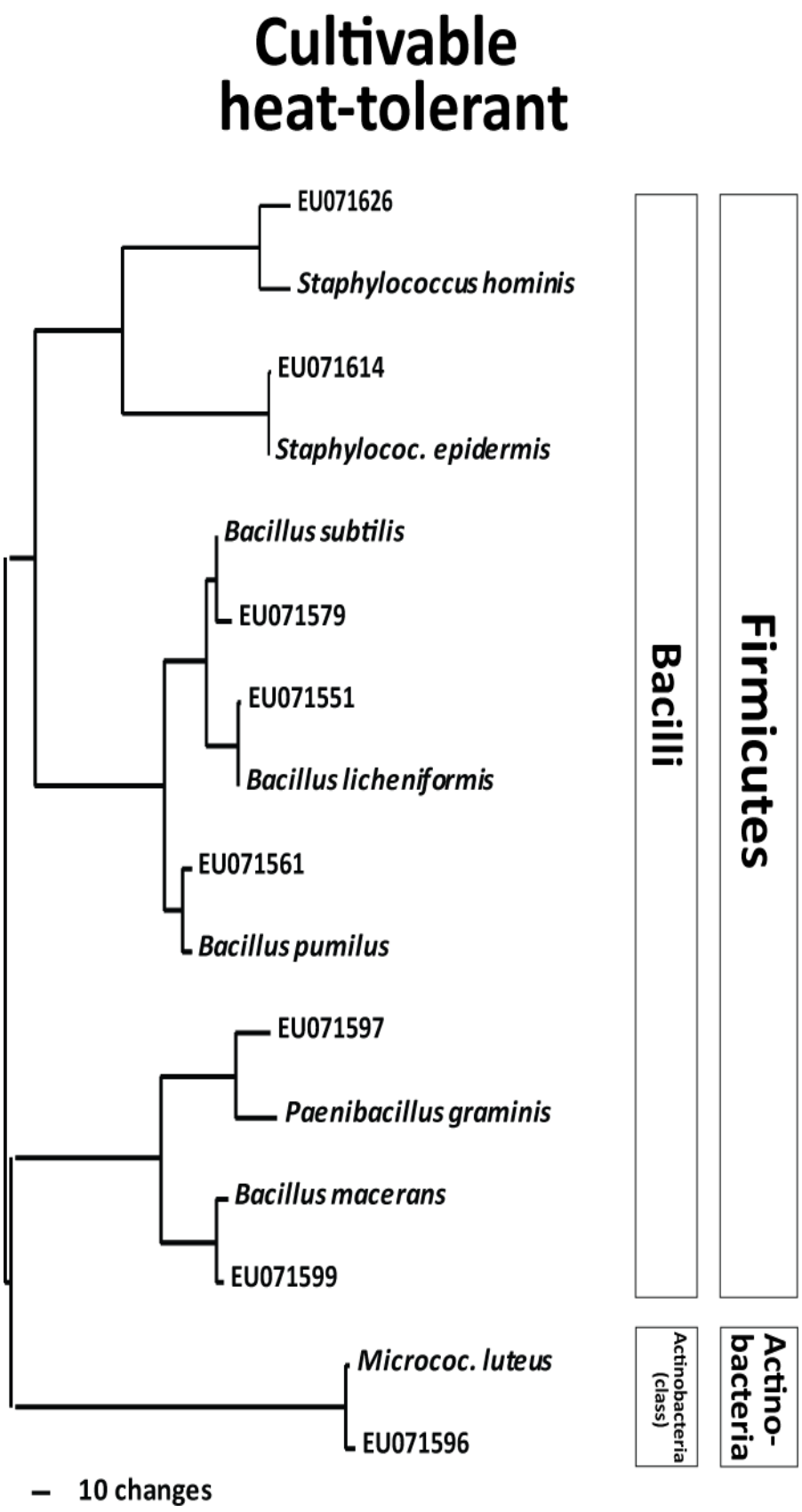

Figure 29: Phylogenetic tree of the identified cultivable heat tolerant bacterial strains.
This Figure is also stored on the Data-CD.

Table 18: Distance-matrix of the identified cultivable heat tolerant bacterial strains.
This Table is also stored on the Data-CD.

	EU071626	EU071597	Bacillus subtilis DSM10	EU071579	EU071614	EU071561	Bacillus macerans	EU071599	Bacillus pumilus DSMZ27	Micrococcus luteus	EU071596	Staphylococcus hominis	Staphylococcus epidermins	EU071551	Bacillus licheniformis	Paenibacillus graminis
EU071626	1,00															
EU071597	85,78	1,00														
Bacillus subtilis DSM10	91,74	86,43	1,00													
EU071579	91,15	85,83	99,41	1,00												
EU071614	98,38	85,78	91,66	91,07	1,00											
EU071561	91,66	87,75	97,05	96,46	92,40	1,00										
Bacillus macerans	85,97	93,63	87,38	86,78	86,18	87,89	1,00									
EU071599	85,91	93,67	87,45	86,86	85,97	87,88	99,25	1,00								
Bacillus pumilus DSMZ27	91,89	87,81	96,98	96,39	92,62	99,41	88,02	88,02	1,00							
Micrococcus luteus	80,84	79,84	81,68	81,08	80,84	81,92	80,86	80,78	81,97	1,00						
EU071596	80,69	79,61	81,45	80,85	80,69	81,84	80,55	80,56	81,90	99,47	1,00					
Staphylococcus hominis	99,85	85,79	91,74	91,16	98,38	91,66	85,98	85,92	91,89	80,85	80,70	1,00				
Staphylococcus epiderminis	98,46	85,86	91,74	91,16	99,93	92,48	86,27	86,06	92,70	80,85	80,71	98,46	1,00			
EU071551	91,37	86,34	98,31	97,72	91,74	96,24	87,59	87,58	96,23	81,28	81,20	91,37	91,81	1,00		
Bacillus licheniformis	91,31	86,28	98,38	97,79	91,67	96,17	87,60	87,59	96,16	81,29	81,22	91,31	91,75	99,93	1,00	
Paenibacillus graminis	85,63	97,06	86,41	85,82	85,69	87,58	93,47	93,37	87,64	79,51	79,21	85,63	85,78	86,32	86,26	1,00

Table 19: Taxonomic information of the identified uncultivable bacterial species.
This Table is also stored on the Data-CD.

Taxonomic information of uncultivable bacterial species I												
GenBank Nr.	Closest species (16S rDNA)	Similarity (%)	Superkingdom	Phylum	Class	Sub-class	Order	Suborder	Family	Genus	Species	Sub-species
EU071537	*Acinetobacter johnsonii* (T) DSM 6963	99,11	Bacteria	Proteobacteria	Gammaproteobacteria		Pseudomonadales		Moraxellaceae	Acinetobacter		
EU071522	*Acinetobacter junii* (T) DSM 6964	99,86	Bacteria	Proteobacteria	Gammaproteobacteria		Pseudomonadales		Moraxellaceae	Acinetobacter		
EU071491	*Acinetobacter lwoffii* (T) DSM 2403	99,32	Bacteria	Proteobacteria	Gammaproteobacteria		Pseudomonadales		Moraxellaceae	Acinetobacter		
EU071474	*Actinomyces odontolyticus* (T) NCTC 9935	99,37	Bacteria	Actinobacteria	Actinobacteria (class)	Actinobacteridae	Actinomycetales	Actinomycineae	Actinomycetaceae	Actinomyces		
EU071503	*Actinomycete Lp2*	97,51	Bacteria	Actinobacteria	Actinobacteria (class)	Actinobacteridae	Actinomycetales	Micrococcineae	Intrasporangiaceae	Tetrasphaera		
EU071489	*Alpha proteobacterium 34632*	99,86	Bacteria	Proteobacteria	Alphaproteobacteria		Rhizobiales		Bradyrhizobiaceae	Afipia		
EU071538	*Amaricoccus macauensis* (T) Ben104	97,77	Bacteria	Proteobacteria	Alphaproteobacteria		Rhodobacterales		Rhodobacteraceae	Amaricoccus		
EU071532	*Bacillus bataviensis* (T) type strain: LMG 21833	98,52	Bacteria	Firmicutes	Bacilli		Bacillales		Bacillaceae	Bacillus		
EU071493	*Bacillus benzoevorans* (T) DSM5391	97,9	Bacteria	Firmicutes	Bacilli		Bacillales		Bacillaceae	Bacillus		
EU071487	*Bacillus subtilis* (T) IAM 12118T	99,47	Bacteria	Firmicutes	Bacilli		Bacillales		Bacillaceae	Bacillus		
EU071523	*Beta proteobacterium B8*	98,29	Bacteria	Proteobacteria	Betaproteobacteria		Burkholderiales			Aquabacterium		
EU071509	*Bifidobacterium ruminantium* (T) JCM8222	97,79	Bacteria	Actinobacteria	Actinobacteria (class)	Actinobacteridae	Bifidobacteriales		Bifidobacteriaceae	Bifidobacterium		
EU071528	*Burkholderia fungorum* (T) LMG 16225	100	Bacteria	Proteobacteria	Betaproteobacteria		Burkholderiales		Burkholderiaceae	Burkholderia		
EU071505	*Catenibacterium mitsuokai* (T) JCM 10609	97,72	Bacteria	Firmicutes	Clostridia		Clostridiales		Lachnospiraceae	Catenibacterium		
EU071485	*Cellulomonas gelida* (T) DSM 20111T	98,27	Bacteria	Actinobacteria	Actinobacteria (class)	Actinobacteridae	Actinomycetales	Micrococcineae	Cellulomonadaceae	Cellulomonas		
EU071533	*Clostridium disporicum* (T) DSM 5521	98,77	Bacteria	Firmicutes	Clostridia		Clostridiales		Clostridiaceae	Clostridium		
EU071510	*Clostridium lituseburense* (T)	97,3	Bacteria	Firmicutes	Clostridia		Clostridiales		Clostridiaceae	Clostridium		
EU071529	*Corynebacterium coyleae* (T) DSM 44184	97,32	Bacteria	Actinobacteria	Actinobacteria (class)	Actinobacteridae	Actinomycetales	Corynebacterineae	Corynebacteriaceae	Corynebacterium		
EU071515	*Corynebacterium kroppenstedtii* (T) CCUG 35717	99,36	Bacteria	Actinobacteria	Actinobacteria (class)	Actinobacteridae	Actinomycetales	Corynebacterineae	Corynebacteriaceae	Corynebacterium		
EU071498	*Corynebacterium tuberculostearicum* (T) CIP107291	99,73	Bacteria	Actinobacteria	Actinobacteria (class)	Actinobacteridae	Actinomycetales	Corynebacterineae	Corynebacteriaceae	Corynebacterium		
EU071506	*Deinococcus radiopugnans* (T) ATCC 19172T	97,75	Bacteria	Deinococcus-Thermus	Deinococci		Deinococcales		Deinococcaceae	Deinococcus		
EU071542	*Filifactor alocis* (T) ATCC 35896	99,32	Bacteria	Firmicutes	Clostridia		Clostridiales		Peptostreptococcaceae	Filifactor		
EU071494	*Flavimonas oryzihabitans*	99,06	Bacteria	Proteobacteria	Gammaproteobacteria		Pseudomonadales		Pseudomonadaceae	Pseudomonas	Pseudomonas putida group	
EU071543	*Fusobacterium canifelinum* (T) RMA 12708 Q11	98,71	Bacteria	Fusobacteria	Fusobacteria (class)		Fusobacteriales		Fusobacteriaceae	Fusobacterium		
EU071516	*Gemella haemolysans* (T)	99,87	Bacteria	Firmicutes	Bacilli		Bacillales		Staphylococcaceae	Gemella		
EU071466	*Gemella morbillorum* (T)	99	Bacteria	Firmicutes	Bacilli		Bacillales		Staphylococcaceae	Gemella		
EU071472	*Gemella sanguinis* (T) 2045-94	99,73	Bacteria	Firmicutes	Bacilli		Bacillales		Staphylococcaceae	Gemella		
EU071514	*Gram-positive bacterium* MOL 361	99,05	Bacteria	Firmicutes	Bacilli		Bacillales		Turicibacteraceae	Turicibacter		
EU071475	*Granulicatella adiacens* (T) GIFU12706	99,22	Bacteria	Firmicutes	Bacilli		Bacillales		Staphylococcaceae	Gemella		
EU071476	*Haemophilus parainfluenzae* (T)	97,57	Bacteria	Proteobacteria	Gammaproteobacteria		Pasteurellales		Pasteurellaceae	Haemophilus		
EU071511	*Haemophilus paraphrohaemolyticus* (T)	97,41	Bacteria	Proteobacteria	Gammaproteobacteria		Pasteurellales		Pasteurellaceae	Haemophilus		
EU071507	*Hymenobacter roseosalivarius* (T) AA718	98,91	Bacteria	Bacteroidetes	Sphingobacteria		Sphingobacteriales		Flexibacteraceae	Hymenobacter		
EU071499	*Knoellia sinensis* (T) DSM 12331	98,78	Bacteria	Actinobacteria	Actinobacteria (class)	Actinobacteridae	Actinomycetales	Micrococcineae	Intrasporangiaceae	Knoellia		
EU071500	*Knoellia subterranea* (T) DSM 12332	99,52	Bacteria	Actinobacteria	Actinobacteria (class)	Actinobacteridae	Actinomycetales	Micrococcineae	Intrasporangiaceae	Knoellia		
EU071482	*Lactobacillus sp.* (T) CCUG 28746	99,76	Bacteria	Firmicutes	Bacilli		Lactobacillales		Lactobacillaceae	Lactobacillus		
EU071483	*Leptolyngbya sp.* PCC 7104 (T)	97,9	Bacteria	Cyanobacteria			Oscillatoriales			Leptolyngbya		
EU071539	*Megasphaera micronuciformis* (T) AIP 412.00 CIP 107280	99,57	Bacteria	Firmicutes	Clostridia		Clostridiales		Acidaminococcaceae	Megasphaera		
EU071540	*Methylobacterium fujisawaense* (T) DSM 5686	98,82	Bacteria	Proteobacteria	Alphaproteobacteria		Rhizobiales		Methylobacteriaceae	Methylobacterium		
EU071518	*Micrococcus lylae* (T) DSM 20315	97,15	Bacteria	Actinobacteria	Actinobacteria (class)	Actinobacteridae	Actinomycetales		Micrococcineae	Micrococcaceae		
EU071517	*Micrococcus psychrophilum*	97,53	Bacteria	Actinobacteria	Actinobacteria (class)	Actinobacteridae	Actinomycetales		Micrococcineae	Micrococcaceae		
EU071467	*Mogibacterium neglectum* (T) ATCC 700924	99,12	Bacteria	Firmicutes	Clostridia		Clostridiales		Eubacteriaceae	Mogibacterium		
EU071480	*Mycobacterium chitae* (T)	98,6	Bacteria	Actinobacteria	Actinobacteria (class)	Actinobacteridae	Actinomycetales	Corynebacterineae	Mycobacteriaceae	Mycobacterium		
EU071524	*Neisseria canis* (T)	98,21	Bacteria	Proteobacteria	Betaproteobacteria		Neisseriales		Neisseriaceae	Neisseria		
EU071525	*Neisseria polysaccharea* (T)	98,3	Bacteria	Proteobacteria	Betaproteobacteria		Neisseriales		Neisseriaceae	Neisseria		
EU071473	*Nocardioides sp.* JC2055	97,89	Bacteria	Actinobacteria	Actinobacteria (class)	Actinobacteridae	Actinomycetales	Propionibacterineae	Nocardioidaceae	Nocardioides		
EU071473	*Novosphingobium aromaticivorans* (T) SMCC F199	97,16	Bacteria	Proteobacteria	Alphaproteobacteria		Sphingomonadales		Sphingomonadaceae	Novosphingobium		
EU071470	*Oribacterium sinus* (T) AIP 354.02	99,34	Bacteria	Firmicutes	Clostridia		Clostridiales		Lachnospiraceae	Oribacterium		
EU071512	*Paracoccus carotinifaciens* (T) E-396	99,56	Bacteria	Proteobacteria	Alphaproteobacteria		Rhodobacterales		Rhodobacteraceae	Paracoccus		
EU071513	*Paracoccus haeundaensis* (T) BC74171	99,88	Bacteria	Proteobacteria	Alphaproteobacteria		Rhodobacterales		Rhodobacteraceae	Paracoccus		
EU071465	*Peptostreptococcus anaerobius* (T) ATCC 27337	98,09	Bacteria	Firmicutes	Clostridia		Clostridiales		Peptostreptococcaceae	Peptostreptococcus		
EU071526	*Prevotella denticola* (T)	97,13	Bacteria	Bacteroidetes	Bacteroidetes (class)		Bacteroidales		Prevotellaceae	Prevotella		
EU071492	*Pseudomonas pseudoalcaligenes* (T) LMG 1225T	99,89	Bacteria	Proteobacteria	Gammaproteobacteria		Pseudomonadales		Pseudomonadaceae	Pseudomonas	Pseudomonas aeruginosa group	
EU071502	*Pseudomonas rhizosphaerae* (T) IH5	98,33	Bacteria	Proteobacteria	Gammaproteobacteria		Pseudomonadales		Pseudomonadaceae	Pseudomonas		
EU071486	*Rickettsia honei* (T) RB	99,11	Bacteria	Proteobacteria	Alphaproteobacteria		Rickettsiales	Rickettsiaceae	Rickettsieae	Rickettsia	spotted fever group	
EU071477	*Rothia aeria* (T) GTC867	99,55	Bacteria	Actinobacteria	Actinobacteria (class)	Actinobacteridae	Actinomycetales	Micrococcineae	Micrococcaceae	Rothia		
EU071468	*Rothia dentocariosa* (T) ATCC 17931	99,25	Bacteria	Actinobacteria	Actinobacteria (class)	Actinobacteridae	Actinomycetales	Micrococcineae	Micrococcaceae	Rothia		
EU071484	*Sphingomonas mali* (T) IFO 10550-T	97,54	Bacteria	Proteobacteria	Alphaproteobacteria		Sphingomonadales		Sphingomonadaceae	Sphingomonas		
EU071479	*Sphingomonas melonis* (T) PG-224	97,89	Bacteria	Proteobacteria	Alphaproteobacteria		Sphingomonadales		Sphingomonadaceae	Sphingomonas		

Taxonomic information of uncultivable bacterial species II

GenBank Nr.	Closest species (16S rDNA)	Similarity (%)	Superkingdom	Phylum	Class	Sub-class	Order	Suborder	Family	Genus	Species	Sub-species
EU071495	*Staphylococcus aureus* (T)	99,73	Bacteria	Firmicutes	Bacilli		Bacillales		Staphylococcaceae	Staphylococcus		
EU071519	*Staphylococcus caprae* (T) ATCC 35538T	99,67	Bacteria	Firmicutes	Bacilli		Bacillales		Staphylococcaceae	Staphylococcus		
EU071544	*Staphylococcus cohnii* (T) ATCC 49330T	97,86	Bacteria	Firmicutes	Bacilli		Bacillales		Staphylococcaceae	Staphylococcus		
EU071534	*Staphylococcus epidermidis* (T) ATCC 14990T	99,73	Bacteria	Firmicutes	Bacilli		Bacillales		Staphylococcaceae	Staphylococcus		
EU071496	*Staphylococcus haemolyticus* (T) ATCC 29970T	99,8	Bacteria	Firmicutes	Bacilli		Bacillales		Staphylococcaceae	Staphylococcus		
EU071530	*Staphylococcus hominis* (T) DSM 20328	99,54	Bacteria	Firmicutes	Bacilli		Bacillales		Staphylococcaceae	Staphylococcus	Staphylococcus hominis	
EU071535	*Staphylococcus saccharolyticus* (T)	100	Bacteria	Firmicutes	Bacilli		Bacillales		Staphylococcaceae	Staphylococcus		
EU071508	*Staphylococcus warneri* (T)	99,73	Bacteria	Firmicutes	Bacilli		Bacillales		Staphylococcaceae	Staphylococcus		
EU071481	*Stenotrophomonas maltophilia* (T) LMG 958-T	99,88	Bacteria	Proteobacteria	Gammaproteobacteria		Xanthomonadales		Xanthomonadaceae	Stenotrophomonas		
EU071497	*Streptococcus australis* (T) ATCC 700641	99,18	Bacteria	Firmicutes	Bacilli		Lactobacillales		Streptococcaceae	Streptococcus		
EU071501	*Streptococcus cremoris*	99,19	Bacteria	Firmicutes	Bacilli		Lactobacillales		Streptococcaceae	Lactococcus	Lactococcus lactis	Lactococcus lactis subsp. cremoris
EU071531	*Streptococcus defectivus*	99,22	Bacteria	Firmicutes	Bacilli		Lactobacillales		Aerococcaceae	Abiotrophia		
EU071520	*Streptococcus mitis* (T) ATCC 49456	100	Bacteria	Firmicutes	Bacilli		Lactobacillales		Streptococcaceae	Streptococcus		
EU071498	*Streptococcus parasanguinis* (T) ATCC 15912	99,6	Bacteria	Firmicutes	Bacilli		Lactobacillales		Streptococcaceae	Streptococcus		
EU071541	*Streptococcus pneumoniae* (T) ATCC 33400	99,79	Bacteria	Firmicutes	Bacilli		Lactobacillales		Streptococcaceae	Streptococcus.		
EU071521	*Streptococcus sanguinis* (T) ATCC 10556	99,73	Bacteria	Firmicutes	Bacilli		Lactobacillales		Streptococcaceae	Streptococcus		
EU071536	*Streptococcus sinensis* (T) HKU4	99,8	Bacteria	Firmicutes	Bacilli		Lactobacillales		Streptococcaceae	Streptococcus		
EU071490	*Streptococcus thermophilus* (T) DSM 20617	99,14	Bacteria	Firmicutes	Bacilli		Lactobacillales		Streptococcaceae	Streptococcus		
EU071527	*Thermomonas brevis* (T) type strain: LMG 21746	97,3	Bacteria	Proteobacteria	Gammaproteobacteria		Xanthomonadales		Xanthomonadaceae	Thermomonas		
EU071504	*Variovorax paradoxus* (T) DSM 66	98,77	Bacteria	Proteobacteria	Betaproteobacteria		Burkholderiales		Comamonadaceae	Variovorax		
EU071469	*Veillonella atypica*	99,27	Bacteria	Firmicutes	Clostridia		Clostridiales		Acidaminococcaceae	Veillonella		
EU071478	*Veillonella parvula* (T) DSM 2008	98,73	Bacteria	Firmicutes	Clostridia		Clostridiales		Acidaminococcaceae	Veillonella		

Table 20: Taxonomic information of the identified cultivable bacterial species.

This Table is also stored on the Data-CD.

Taxonomic information cultivable bacterial species I

GenBank Nr.	Closest species (16S rDNA)	Similarity (%)	Superkingdom	Phylum	Class	Sub-class	Order	Suborder	Family	Genus	Species	Sub-species
EU071546	*Bacillus atrophaeus* (T), JCM9070	99,86	Bacteria	Firmicutes	Bacilli		Bacillales		Bacillaceae	Bacillus		
EU071548	*Bacillus flexus* (T), IFO15715	99,14	Bacteria	Firmicutes	Bacilli		Bacillales		Bacillaceae	Bacillus		
EU071549	*Bacillus licheniformis* (T), DSM 13	99,65	Bacteria	Firmicutes	Bacilli		Bacillales		Bacillaceae	Bacillus		
EU071557	*Bacillus muralis* (T), type strain: LMG 20238	99,57	Bacteria	Firmicutes	Bacilli		Bacillales		Bacillaceae	Bacillus		
EU071561	*Bacillus pumilus* (T), DSMZ27	99,15	Bacteria	Firmicutes	Bacilli		Bacillales		Bacillaceae	Bacillus		
EU071573	*Bacillus subtilis* (T), DSM10	100	Bacteria	Firmicutes	Bacilli		Bacillales		Bacillaceae	Bacillus		
EU071588	*Bacillus vallismortis* (T), DSM11031	99,5	Bacteria	Firmicutes	Bacilli		Bacillales		Bacillaceae	Bacillus		
EU071589	*Corynebacterium flavescens* (T), NCDO 1320	99,78	Bacteria	Actinobacteria	Actinobacteria (class)	Actinobacteridae	Actinomycetales	Corynebacterineae	Corynebacteriaceae	Corynebacterium		
EU071591	*Micrococcus luteus* (T), type strain: DSM 20030	99,71	Bacteria	Anctinobacteria	Actinobacteria (class)	Actinobacteridae	Actinomycetales		Micrococcineae	Micrococcaceae		
EU071598	*Paenibacillus graminis* (T), RSA19	97,11	Bacteria	Firmicutes	Bacilli		Bacillales		Paenibacillaceae	Paenibacillus		
EU071599	*Paenibacillus macerans* (T), DSM 24	97,47	Bacteria	Firmicutes	Bacilli		Bacillales		Paenibacillaceae	Paenibacillus		
EU071600	*Pseudomonas stutzeri* (T), CCUG 11256	97,03	Bacteria	Proteobacteria	Gammaproteobacteria		Pseudomonadales		Pseudomonadaceae	Pseudomonas	*Pseudomonas stutzeri* group	
EU071601	*Roseomonas mucosa* (T), MDA5527	100	Bacteria	Proteobacteria	Alphaproteobacteria		Rhizobiales		Methylobacteriaceae	Roseomonas		
EU071602	*Staphylococcus aureus* (T)	99,93	Bacteria	Firmicutes	Bacilli		Bacillales		Staphylococcaceae	Staphylococcus		
EU071603	*Staphylococcus caprae* (T), ATCC 35538T	99,86	Bacteria	Firmicutes	Bacilli		Bacillales		Staphylococcaceae	Staphylococcus		
EU071606	*Staphylococcus epidermidis* (T), ATCC 14990T	99,79	Bacteria	Firmicutes	Bacilli		Bacillales		Staphylococcaceae	Staphylococcus		
EU071615	*Staphylococcus haemolyticus* (T), ATCC 29970T	99,65	Bacteria	Firmicutes	Bacilli		Bacillales		Staphylococcaceae	Staphylococcus		
EU071612	*Staphylococcus hominis* (T), DSM 20328	99,86	Bacteria	Firmicutes	Bacilli		Bacillales		Staphylococcaceae	Staphylococcus	*Staphylococcus hominis*	

Table 21: Significance of the identified uncultivable bacterial species.
This Table is also stored on the Data-CD.

Significance of identified uncultivable bacterial species I

GenBank Nr.	Closest species (16S rDNA)	16S rDNA similarity(%)	Gram stain	Habitat	Significance
EU071537	*Acinetobacter johnsonii (T) DSM 6963*	99,11	Neg.	Env.	Isolated from activated sludge (can cause vascular infections), innately resistant to many classes of antibiotics including penicillin, chloramphenicol and often aminoglycosides
EU071522	*Acinetobacter junii (T) DSM 6964*	99,86	Neg.	Env.	Isolated from soil and water, rare opportunistic pathogen (infections in infants), innately resistant to antibiotics including penicillin, chloramphenicol and often aminoglycosides
EU071491	*Acinetobacter lwoffii (T) DSM 2403*	99,32	Neg.	Env.	Isolated from soil and water, human pathogen (meningitis), innately resistant to many classes of antibiotics including penicillin, chloramphenicol and often aminoglycosides
EU071474	*Actinomyces odontolyticus (T) NCTC 9935*	99,37	Pos.	Hum.	Comensal human flora, opportunistic pathogen (endogenous infections)
EU071503	*Actinomycete Lp2*	97,51	Pos.	Env.	Isolated from activated sludge (Japan), enhanced biological phosphorus removal activity
EU071489	*Alpha proteobacterium_34632*	99,86	Neg.	Env.	requirements)
EU071538	*Amaricoccus macauensis (T) Ben104*	97,77	Neg.	Env.	Isolated from activated sludge from wastewater treatment plant in Macau
EU071532	*Bacillus bataviensis (T) type strain: LMG 21833*	98,52	Pos.	Env.	Isolated from soil (Netherlands)
EU071493	*Bacillus benzoevorans (T) DSM5391*	97,9	Pos.	Env.	Isolated from soil
EU071487	*Bacillus subtilis (T) IAM 12118T*	99,47	Pos.	Env.	Isolated from soil, known to tolerate extreme environmental conditions in endosporefrom
EU071523	*Beta proteobacterium B8*	98,29	Neg.	Env.	Iolated from Berlin drinking water system, one of the three detected dominant bacterial species
EU071509	*Bifidobacterium ruminantium (T) JCM8222*	97,79	Pos.	An.	Isolated fom rumen of cattle
EU071528	*Burkholderia fungorum (T) LMG 16225*	100	Neg.	Env., Hum., An.	Isolated from the environment, animals and human clinical samples (can cause community-acquired bacteremia)
EU071505	*Catenibacterium mitsuokai (T) JCM 10609*	97,72	Pos.	Hum.	Isolated from human faeces
EU071485	*Cellulomonas gelida (T) DSM 20111T*	98,27	Pos.	Env.	Isolated from soil, cellulolytic
EU071533	*Clostridium disporicum (T) DSM 5521*	98,77	Pos.	An.	Isolated from rat cecum
EU071510	*Clostridium lituseburense (T)*	97,3	Pos.	Env.	Isolated from mud
EU071529	*Corynebacterium coyleae (T)*	97,32	Pos.	Hum.	Isolated from human blood culture of immunodeficient patents, opportunistic pathogen
EU071515	*Corynebacterium kroppenstedtii (T) CCUG 35717*	99,36	Pos.	Hum.	Isolated from sternum wound swab
EU071498	*Corynebacterium tuberculostearicum (T) CIP107291*	99,73	Pos.	Hum.	Isolated from clinical samples (Biosaftey level 2)
EU071506	*Deinococcus radiopugnans (T) ATCC 19172T*	97,75	Pos.	Env.	Isolated from irradiated haddock, highly radiation-resistant
EU071542	*Filifactor alocis (T) ATCC 35896*	99,32	Neg.	Hum.	Comensal human flora, opportunistic pathogen (endodontic infections)
EU071494	*Flavimonas oryzihabitans*	99,06	Neg.	Env.	Isolated from rice paddy (can cause peritonitis)
EU071543	*Fusobacterium canifelinum (T) RMA 12708 Q11*	98,71	Neg.	An.	Isolated from oral cavities of cats and dogs
EU071516	*Gemella haemolysans (T)*	99,87	Pos.	Hum.	Comensal human flora, opportunistic pathogen (endovascular infections)
EU071466	*Gemella morbillorum (T)*	99	Pos.	Hum.	Comensal human flora, opportunistic pathogen (predominantly endocarditis)
EU071472	*Gemella sanguinis (T) 2045-94*	99,73	Pos.	Hum.	Comensal human flora, opportunistic pathogen (endovascular infections)
EU071514	*Gram-positive_bacterium_MOL361*	99,05	Pos.	Hum.	Isolated from human blood (can cause accutre appendicitis), anaerobic
EU071475	*Granulicatella adiacens (T) GIFU12706*	99,22	Pos.	Hum.	Comensal human flora, opportunistic pathogen (endovascular infections)
EU071476	*Haemophilus parainfluenzae (T)*	97,57	Neg.	Hum.	Comensal human flora, opportunistic pathogen (endovascular infections)
EU071511	*Haemophilus paraphrohaemolyticus (T)*	97,41	Neg.	Hum.	Comensal human flora, opportunistic pathogen (liver abscess)
EU071507	*Hymenobacter roseosalivarius (T) AA718*	98,91	Neg.	Env.	Isolated from antartic soil and sandstone
EU071499	*Knoellia sinensis (T) DSM 12331*	98,78	Pos.	Env.	Isolated from a cave in China
EU071500	*Knoellia subterranea (T) DSM 12332*	99,52	Pos.	Env.	Isolated from a cave in China
EU071482	*Lactobacillus sp. (T) CCUG 28746*	99,76	Pos.	Hum.	Comensal human flora, opportunistic pathogen (caries), high hydrogen peroxide tolerance
EU071483	*Leptolyngbya sp. PCC 7104 (T)*	97,9	Neg.	Env.	Isollated from cristal waters, copper resistant
EU071539	*Megasphaera micronuciformis (T) AIP 412.00*	99,57	Neg.	Hum.	Isolated from human clinical sample (liver abscess)
EU071540	*Methylobacterium fujisawaense (T) DSM 5686*	98,82	Neg.	Env.	Isolated from plant
EU071518	*Micrococcus lylae (T) DSM 20315*	97,15	Pos.	Hum.	Comensal human flora (skin), rare opportunistic pathogen (endocarditis), associated with spoilage of fish products
EU071517	*Micrococcus psychrophilum*	97,53	Pos.	Env.	Isolated from Antarctica, psychrophilic
EU071467	*Mogibacterium neglectum (T) TCC 700924*	99,12	Pos.	Hum.	Isolated from tongue plaque and necrotic dental pulp, anaerobic
EU071480	*Mycobacterium chitae (T)*	98,6	Pos.	Env.	Isolated from soil
EU071524	*Neisseria canis (T)*	98,21	Neg.	An.	Isolated from oral mucosa of dog
EU071525	*Neisseria polysaccharea (T)*	98,3	Neg.	Hum.	Comensal human flora, can carry 3-Lactam resistance

Significance of identified uncultivable bacterial species II

GenBank Nr.	Closest species (16S rDNA)	16S rDNA similarity(%)	Gram stain	Habitat	Significance
EU071473	*Nocardioides sp. JC2055*	97,89	Pos.	Env.	Isolated from tidal flat sediment sample of the getbol of Ganghwa island
EU071473	*Novosphingobium aromaticivorans (T) SMCC F199*	97,16	Neg.	Env.	Isolated from subsurface cretaceous age formation at 410 m depth, capable of growth on toluene, naphtalene and other aromatic compounds
EU071470	*Oribacterium sinus (T) AIP 354.02*	99,34	Pos.	Hum.	Isolated from human, maxillary sinus
EU071512	*Paracoccus carotinifaciens (T) E-396*	99,56	Neg.	Env.	Isolated from soil, astaxanthin-producing
EU071513	*Paracoccus haeundaensis (T) BC74171*	99,88	Neg.	Env.	Isolated from Haeundae Coast
EU071465	*Peptostreptococcus anaerobius (T) ATCC 27337*	98,09	Pos.	Hum.	Comensal human flora, opportunistic pathogen (Risk Group 2) (systemic infections including endocarditis), vancomycin sensitive, colistin and kanamycin resistant
EU071526	*Prevotella denticola (T)*	97,13	Neg.	Hum.	Isolated from dental plaque
EU071492	*Pseudomonas pseudoalcaligenes (T) LMG 1225T*	99,89	Neg.	Env.	Isolated from water, can carry tetracycline resistance genes (*tet* gene)
EU071502	*Pseudomonas rhizosphaerae (T) IH5*	98,33	Neg.	Env.	Isolated from rhizosphere of grasses
EU071486	*Rickettsia honei (T) RB*	99,11	Neg.	Insect	Isolated from ticks, human pathogen causes spotted fever
EU071477	*Rothia aeria (T) GTC867*	99,55	Pos.	Hum.	Isolated from MIR spacestation
EU071468	*Rothia dentocariosa (T) ATCC 17931*	99,25	Pos.	Hum.	Isolated from carious teeth, opportunistic pathogen (Risk group 2) (subacute infective endocarditis)
EU071484	*Sphingomonas mali (T) IFO 10550-T*	97,54	Neg.	Env.	Isolated from roots of apple tree
EU071479	*Sphingomonas melonis (T) PG-224*	97,89	Neg.	Env.	Isolated from fruits of yellow spanish melons, plant pathogen (causes brown spots on yellow spanish melon fruits)
EU071495	*Staphylococcus aureus (T)*	99,73	Pos.	Hum.	Comensal human flora, can act as opportunistic pathogen (wide range of infections), antibiotic resistance (MRSA) known
EU071519	*Staphylococcus caprae (T) ATCC 35538T*	99,67	Pos.	Hum., An.	Isolated from goats and humans, opportunistic pathogen (dermatitis)
EU071544	*Staphylococcus cohnii (T) ATCC 49330T*	97,86	Pos.	Hum.	Comensal human flora (skin), rare opportunistic pathogen (for humans causing urinary tract infections, wound infections, endocarditis and septicemia)
EU071534	*Staphylococcus epidermidis (T) ATCC 14990T*	99,73	Pos.	Hum.	Comensal human flora (skin), opportunistic pathogen (major agents of nosocomial infections), often resistant to antibiotics (penicillin and methicillin)
EU071496	*Staphylococcus haemolyticus (T) ATCC 29970T*	99,8	Pos.	Hum.	Comensal human flora (skin), rare opportunistic pathogen, resistance to vancomycin has been recorded
EU071530	*Staphylococcus hominis (T) DSM 20328*	99,54	Pos.	Hum.	Isolated from human blood culture, opportunistic pathogen (Risk group 2), multiple-antibiotic-resistant subspecies isolated
EU071535	*Staphylococcus saccharolyticus (T)*	100	Pos.	Hum.	Isolated from human plasma, opportunistic pathogen (Risk group 2) (anaerobic endocarditis)
EU071508	*Staphylococcus warneri (T)*	99,73	Pos.	Hum.	Comensal human flora (skin), rare opportunistic pathogen
EU071481	*Stenotrophomonas maltophilia (T) LMG 958-T*	99,88	Neg.	Env.	Isolated from ubiquitous in aqueous environments, soil and plants, human pathogen (Risk group 2), naturally resistant to many broad-spectrum antibiotics
EU071497	*Streptococcus australis (T) ATCC 700641*	99,18	Pos.	Hum.	Isolated from saliva from children, opportunistic pathogen (Risk group 2), facultative anaerobic
EU071520	*Streptococcus mitis (T) ATCC 49456*	100	Pos.	Hum.	Comensal human flora (oral), opportunistic pathogen (Risk group 2) (endocarditis)
EU071471	*Streptococcus parasanguinis (T) ATCC 15912*	99,6	Pos.	Hum.	Comensal human flora (throat), opportunistic pathogen (Risk group 2)
EU071541	*Streptococcus pneumoniae (T) ATCC 33400*	99,79	Pos.	Hum.	Comensal human flora, opportunistic pathogen (Risk group 2) (major for cause pneumonia)
EU071521	*Streptococcus sanguinis (T) ATCC 10556*	99,73	Pos.	Hum.	Comensal human flora (oral), opportunistic pathogen (Risk group 2) (common cause of subacute bacterial endocarditis)
EU071536	*Streptococcus sinensis (T) HKU4*	99,8	Pos.	Hum.	Isolated from human blood sample, human pathogen (Risk group 2) (infective endocarditis)
EU071490	*Streptococcus thermophilus (T) DSM 20617*	99,14	Pos.	dairy	Isolated from pasteurized milk
EU071501	*Streptococcus cremoris*	99,19	Pos.	Hum.	Comensal human flora, high hydrogen peroxide tolerance, cheese production
EU071531	*Streptococcus defectivus*	99,22	Pos.	Hum.	Isolated from humans, human pathogen (mayor cause for endocarditis)
EU071527	*Thermomonas brevis (T) type strain: LMG 21746*	97,3	Neg.	Env.	Isolated from denitrification reactor biofilm
EU071504	*Variovorax paradoxus (T) DSM 66*	98,77	Neg.	Env.	Isolated from soil
EU071478	*Veillonella parvula (T) DSM 2008*	98,73	Neg.	Hum.	Comensal human flora (oral), rare opportunistic pathogen (Risk group 2) (osteomyelitis)
EU071469	*Veillonella atypica*	99,27	Neg.	Hum.	Comensal human flora (oral), early colonizing members of the dental plaque biofilm

An. = animal, dairy. = dairy product, Env. = environmental, Hum. = human,

Table 22: Significance of the identified cultivable bacterial species.
This Table is also stored on the Data-CD.

Significance of identified cultivable bacterial species I

GenBank Nr.	Closest species (16S rDNA)	Gram stain	Habitat	Significance
EU071546	*Bacillus atrophaeus* (T) JCM9070	Pos.	Env.	Isolated from soil, known to tolerate extreme environmental conditions in endosporefrom
EU071548	*Bacillus flexus* (T) IFO15715	Pos.	Env.	Isolated from soil, known to tolerate extreme environmental conditions in endosporefrom
EU071555	*Bacillus licheniformis* (T) DSM 13	Pos.	Env.	Isolated from soil, known to tolerate extreme environmental conditions in endosporefrom
EU071557	*Bacillus muralis* (T) type strain: LMG 20238	Pos.	Env.	Isolated from mural painting
EU071561	*Bacillus pumilus* (T) DSMZ27	Pos.	Env.	Isolated from spacecraft associated environment, highly resistant strain
EU071573	*Bacillus subtilis* (T) DSM10	Pos.	Env.	Isolated from soil, known to tolerate extreme environmental conditions in endosporefrom
EU071588	*Bacillus vallismortis* (T) DSM11031	Pos.	Env.	Isolated from soil in Death Valley
EU071589	*Corynebacterium flavescens* (T) NCDO 1320	Pos.	Dairy	Isolated from cheese
EU071591	*Micrococcus luteus* (T) type strain: DSM 20030	Pos.	Hum.	Comensal human flora (nasal), opportunistic pathogen, known for various resistances
EU071598	*Paenibacillus graminis* (T) RSA19	Pos.	Env.	Isolated from plant roots, soil and food
EU071599	*Paenibacillus macerans* (T) DSM 24	Pos.	Env.	Isolated from soil and from the rhizosphere
EU071600	*Pseudomonas stutzeri* (T) CCUG 11256	Neg.	Env.	Isolated (once) from a silver mine, silver resistant (plasmid)
EU071601	*Roseomonas mucosa* (T) ATCC BAA-692	Neg.	Env., Hum.	Isolated from blood, wounds, exudates, water enviroments
EU071602	*Staphylococcus aureus* (T)	Pos.	Hum.	Comensal human flora, can act as opportunistic pathogen (wide range of infections), antibiotic resistance (MRSA) known
EU071603	*Staphylococcus capitis (T)*	Pos.	Hum., An.	Isolated from goats and humans, opportunistic pathogen (dermatitis)
EU071606	*Staphylococcus epidermidis* (T) ATCC 14990T	Pos.	Hum.	Comensal human flora (skin), opportunistic pathogen (major agents of nosocomial infections), often resistant to antibiotics (penicillin and methicillin)
EU071615	*Staphylococcus haemolyticus* (T) ATCC 29970T	Pos.	Hum.	Comensal human flora (skin), rare opportunistic pathogen, resistant to vancomycin has been recorded
EU071622	*Staphylococcus hominis* (T) DSM 20328	Pos.	Hum.	Isolated from human blood culture, opportunistic pathogen (Risk group 2), multiple-antibiotic-resistant subspecies isolated

An. = animal, dairy. = dairy product, Env. = environmental, Hum. = human,

Table 23: Growth of the tested bacteria strains under the different treatments.
This Table is also stored on the Data-CD.

	Antibiotics						Heavy metals						Biocides				Environmental			
GenBank Nr. (closest species)	Penicillin	Ciprofloxacin	Trimetrophin	Rifampin	Nisin	Gramicidin	Mercury	Zinc	Cobalt	Copper	Cadmium	Arsen	Ethanol	Isopropanol	Formaldehyde	Glutaraldehyde	UV-C	1M NaCl	pH 9,4	pH 4	dessication
EU071578 (*Bacillus subtilis*)	2,4%	2,8%	7,3%	1,9%	100,0%	5,0%	5,9%	27,4%	4,5%	8,3%	5,2%	10,7%	3,4%	3,4%	8,2%	14,7%	0,0%	30,2%	61,6%	12,5%	106,7%
EU071616 (*Staphylococcus haemolyticus*)	6,5%	9,8%	66,4%	25,8%	69,1%	14,7%	4,3%	35,4%	13,1%	20,9%	8,9%	74,5%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	5,0%
EU071587 (*Bacillus subtilis*)	95,7%	15,8%	12,2%	15,9%	99,6%	7,6%	11,5%	7,5%	9,3%	10,2%	6,5%	22,8%	5,3%	5,4%	6,5%	14,9%	3,0%	6,8%	74,6%	56,6%	2,1%
EU071562 (*Bacillus subtilis*)	100,0%	12,9%	11,6%	10,9%	78,9%	13,3%	3,4%	5,4%	6,3%	8,8%	0,0%	53,0%	4,1%	4,8%	10,7%	19,8%	0,0%	5,6%	40,9%	77,5%	70,2%
EU071563 (*Bacillus subtilis*)	100,0%	9,5%	16,7%	1,1%	100,0%	13,4%	0,0%	0,0%	2,3%	3,0%	0,0%	30,5%	4,2%	2,9%	3,7%	23,0%	0,0%	26,5%	66,8%	84,8%	98,9%
EU071617 (*Staphylococcus hominis*)	30,3%	20,3%	58,2%	17,8%	6,6%	38,2%	10,0%	2,8%	5,0%	10,9%	5,8%	6,7%	11,8%	10,8%	11,0%	29,3%	0,0%	45,4%	56,9%	24,4%	52,0%
EU071591 (*Micrococcus luteus*)	100,0%	8,3%	8,7%	5,7%	98,2%	3,4%	4,0%	0,0%	3,0%	3,6%	6,8%	20,4%	3,3%	3,1%	6,4%	11,9%	0,0%	15,5%	81,3%	11,6%	-0,5%
EU071607 (*Staphylococcus epidermidis*)	100,0%	28,0%	74,2%	22,4%	28,6%	12,1%	6,9%	6,3%	22,2%	7,6%	7,4%	48,9%	4,1%	1,7%	1,8%	0,0%	8,7%	31,0%	27,9%	2,9%	-1,8%
EU071564 (*Bacillus subtilis*)	41,3%	39,8%	47,2%	45,2%	52,7%	42,8%	27,3%	32,3%	15,3%	23,4%	29,2%	25,2%	18,0%	19,2%	32,8%	46,6%	0,0%	31,9%	61,7%	26,4%	101,8%
EU071565 (*Bacillus subtilis*)	100,0%	9,6%	16,8%	10,9%	93,3%	3,9%	6,5%	6,9%	4,8%	4,9%	5,7%	38,6%	4,9%	3,4%	7,0%	17,1%	0,0%	12,6%	52,2%	4,9%	-3,7%
EU071618 (*Staphylococcus hominis*)	4,9%	10,4%	71,2%	21,3%	1,5%	21,3%	6,9%	6,3%	22,2%	5,3%	2,5%	10,1%	5,0%	0,8%	1,2%	0,0%	0,0%	4,2%	27,8%	1,5%	-2,3%
EU071566 (*Bacillus subtilis*)	100,0%	11,9%	6,8%	14,1%	100,0%	7,0%	0,0%	5,1%	12,5%	20,3%	4,4%	32,2%	3,7%	4,0%	7,4%	19,7%	0,0%	5,0%	41,4%	91,1%	55,0%
EU071567 (*Bacillus subtilis*)	75,9%	3,5%	3,9%	9,5%	85,1%	4,8%	24,0%	23,4%	2,7%	2,9%	8,0%	55,7%	3,3%	4,2%	5,0%	16,9%	0,0%	8,8%	74,9%	87,1%	71,5%
EU071568 (*Bacillus subtilis*)	100,0%	7,7%	10,7%	7,6%	83,5%	4,1%	3,0%	28,1%	3,8%	3,8%	3,8%	13,8%	3,0%	3,3%	6,8%	19,4%	1,9%	5,8%	34,8%	4,4%	47,8%
EU071579 (*Bacillus subtilis*)	98,8%	15,7%	13,3%	19,2%	91,0%	7,1%	9,6%	6,5%	7,8%	8,7%	10,9%	4,9%	5,8%	4,8%	8,9%	20,0%	1,3%	12,0%	81,3%	14,3%	68,7%
EU071569 (*Bacillus subtilis*)	99,7%	13,0%	13,0%	13,1%	100,0%	5,3%	12,7%	1,8%	2,1%	6,6%	6,0%	21,6%	3,0%	3,6%	5,5%	16,5%	0,0%	6,6%	47,5%	100,0%	1,2%
EU071619 (*Staphylococcus hominis*)	4,2%	21,3%	48,9%	15,5%	1,3%	17,9%	5,0%	79,3%	2,5%	10,7%	20,8%	12,2%	5,2%	1,9%	6,7%	0,0%	0,0%	3,8%	26,1%	8,9%	-0,5%
EU071570 (*Bacillus subtilis*)	100,0%	12,5%	12,4%	7,1%	63,1%	5,1%	16,3%	5,0%	2,4%	7,4%	0,0%	27,6%	3,6%	3,7%	4,9%	17,3%	0,0%	8,4%	39,7%	82,0%	30,6%
EU071559 (*Bacillus pumilus*)	1,5%	3,1%	9,4%	4,3%	88,5%	5,1%	0,0%	27,6%	4,9%	1,1%	12,7%	2,5%	4,0%	3,2%	5,6%	14,6%	0,0%	10,5%	61,4%	41,9%	55,0%
EU071548 (*Bacillus flexus*)	100,0%	17,1%	23,5%	23,9%	73,6%	11,4%	7,4%	14,4%	8,4%	15,1%	24,8%	85,6%	7,4%	6,3%	14,1%	24,0%	0,0%	22,8%	66,9%	13,2%	16,1%
EU071549 (*Bacillus flexus*)	97,4%	18,0%	35,0%	24,1%	4,9%	9,7%	4,0%	13,7%	7,7%	13,4%	21,3%	79,1%	7,4%	8,7%	12,1%	26,7%	0,0%	17,5%	69,3%	9,1%	41,6%
EU071620 (*Staphylococcus hominis*)	90,1%	23,9%	58,1%	30,8%	11,7%	8,1%	8,5%	7,0%	7,4%	4,9%	22,7%	66,2%	6,7%	5,6%	7,3%	23,2%	0,0%	57,3%	19,5%	18,8%	9,5%
EU071551 (*Bacillus licheniformis*)	90,6%	2,8%	4,6%	0,1%	100,0%	4,5%	0,2%	1,7%	3,6%	1,9%	2,4%	88,3%	6,8%	8,6%	6,4%	12,1%	0,0%	51,8%	75,8%	16,5%	84,5%
EU071552 (*Bacillus licheniformis*)	86,2%	5,6%	10,0%	1,0%	90,4%	4,3%	2,6%	1,5%	4,0%	4,8%	6,9%	91,3%	3,4%	1,8%	6,5%	12,7%	0,0%	12,7%	83,5%	6,4%	74,3%
EU071571 (*Bacillus subtilis*)	100,0%	28,0%	74,2%	22,4%	28,6%	12,1%	6,9%	6,3%	22,2%	7,6%	7,4%	48,9%	4,1%	1,7%	1,8%	0,0%	8,7%	31,0%	27,9%	2,9%	32,8%
EU071604 (*Staphylococcus epidermidis*)	4,8%	26,4%	43,9%	15,7%	73,3%	12,1%	18,8%	24,9%	14,2%	15,5%	21,2%	68,0%	8,5%	8,7%	14,3%	21,4%	0,0%	73,2%	41,6%	34,3%	52,8%
EU071608 (*Staphylococcus epidermidis*)	100,0%	25,4%	43,7%	100,0%	61,6%	11,9%	7,0%	4,5%	21,5%	5,8%	6,9%	41,0%	2,3%	1,2%	2,5%	0,2%	7,4%	68,0%	39,2%	3,7%	-1,6%
EU071572 (*Bacillus subtilis*)	8,7%	6,5%	4,8%	14,0%	12,1%	30,2%	100,0%	19,0%	12,0%	13,1%	2,7%	6,3%	3,5%	3,8%	6,9%	16,0%	0,0%	7,0%	35,6%	76,5%	0,9%
EU071581 (*Bacillus subtilis*)	100,0%	13,4%	17,3%	12,7%	89,3%	4,8%	8,6%	4,6%	4,2%	4,5%	6,4%	68,6%	4,5%	3,5%	5,0%	16,9%	0,0%	12,3%	45,1%	4,9%	2,1%
EU071573 (*Bacillus subtilis*)	100,0%	11,0%	7,0%	11,0%	88,6%	5,5%	5,4%	5,3%	2,5%	8,1%	6,6%	20,0%	8,1%	4,9%	7,5%	13,1%	0,0%	12,2%	20,7%	80,1%	31,5%
EU071574 (*Bacillus subtilis*)	100,0%	10,9%	27,2%	7,6%	100,0%	12,3%	6,5%	25,4%	3,2%	7,5%	3,2%	50,0%	9,6%	11,2%	12,7%	15,0%	0,0%	20,2%	39,1%	38,7%	3,2%
EU071545 (*Bacillus atrophaeus*)	100,0%	10,9%	27,2%	7,6%	100,0%	12,3%	6,5%	25,4%	3,2%	7,5%	3,2%	50,0%	9,6%	11,2%	12,7%	15,0%	0,0%	20,2%	39,1%	38,7%	3,2%
EU071546 (*Bacillus atrophaeus*)	100,0%	6,8%	23,3%	3,6%	98,5%	6,5%	6,2%	33,3%	3,3%	7,4%	6,8%	49,0%	6,1%	9,1%	11,6%	13,4%	0,0%	18,8%	37,2%	54,2%	3,2%
EU071594 (*Micrococcus luteus*)	4,2%	21,3%	52,2%	19,5%	1,3%	17,7%	3,3%	79,3%	2,5%	10,7%	23,4%	12,2%	3,1%	1,9%	8,3%	9,0%	0,0%	3,8%	21,7%	7,7%	5,0%
EU071582 (*Bacillus subtilis*)	100,0%	9,4%	8,3%	7,6%	100,0%	2,5%	4,3%	11,3%	0,1%	5,8%	2,0%	26,2%	2,6%	2,2%	4,8%	11,0%	0,0%	8,7%	41,4%	9,9%	55,0%
EU071575 (*Bacillus subtilis*)	100,0%	12,1%	10,4%	8,0%	91,6%	6,2%	8,0%	5,8%	7,7%	9,8%	6,8%	34,0%	7,1%	6,7%	8,5%	20,6%	0,0%	10,9%	22,5%	78,3%	3,1%
EU071589 (*Corynebacterium flavescens*)	9,8%	12,2%	22,4%	14,9%	100,0%	11,5%	3,1%	9,4%	5,1%	5,9%	6,3%	42,8%	6,1%	6,7%	8,0%	19,4%	0,0%	18,2%	38,7%	8,4%	3,5%
EU071615 (*Staphylococcus haemolyticus*)	22,7%	23,4%	48,3%	36,6%	80,0%	14,6%	13,0%	72,5%	19,5%	26,9%	26,5%	100,0%	15,9%	14,9%	20,7%	22,3%	0,0%	61,7%	25,7%	52,0%	2,3%
EU071560 (*Bacillus pumilus*)	1,5%	5,9%	19,9%	5,1%	100,0%	4,0%	4,2%	31,7%	19,0%	3,7%	6,7%	39,0%	6,0%	2,9%	3,4%	4,3%	0,0%	9,4%	48,5%	49,4%	1,9%
EU071610 (*Staphylococcus epidermidis*)	84,0%	10,6%	27,8%	92,3%	54,4%	11,1%	7,0%	22,2%	14,1%	15,0%	20,1%	60,5%	11,5%	12,1%	13,5%	11,7%	0,0%	55,5%	41,0%	36,8%	2,4%
EU071611 (*Staphylococcus epidermidis*)	100,0%	20,1%	65,6%	45,0%	45,2%	11,2%	5,4%	6,0%	20,0%	6,7%	7,3%	47,0%	4,5%	4,6%	5,9%	1,6%	0,0%	9,7%	39,0%	4,6%	15,2%
EU071576 (*Bacillus subtilis*)	100,0%	14,3%	10,0%	18,1%	100,0%	7,2%	13,1%	13,0%	7,1%	12,1%	9,3%	38,6%	4,8%	4,1%	7,2%	13,0%	0,0%	4,8%	16,0%	25,5%	2,4%
EU071577 (*Bacillus subtilis*)	100,0%	12,4%	9,4%	14,5%	100,0%	6,4%	11,9%	9,1%	6,9%	9,0%	11,9%	35,7%	4,2%	5,1%	6,9%	19,5%	0,0%	18,5%	100,0%	100,0%	43,8%
EU071612 (*Staphylococcus epidermidis*)	1,8%	6,9%	55,4%	17,7%	78,2%	9,0%	0,0%	7,7%	0,0%	4,8%	3,7%	63,2%	29,1%	11,9%	3,5%	11,0%	0,0%	62,8%	34,0%	12,4%	16,1%
EU071613 (*Staphylococcus epidermidis*)	2,0%	8,1%	58,6%	15,4%	39,8%	0,9%	3,5%	10,2%	2,2%	20,9%	55,8%	68,5%	20,0%	21,2%	9,4%	16,3%	0,0%	58,3%	30,7%	14,0%	5,0%
EU071625 (*Staphylococcus hominis*)	87,9%	19,6%	63,3%	17,8%	7,9%	8,3%	3,0%	7,3%	2,6%	7,4%	0,0%	66,4%	28,7%	8,2%	5,4%	16,5%	0,0%	45,9%	25,9%	8,5%	2,9%
EU071606 (*Staphylococcus epidermidis*)	87,8%	9,5%	41,7%	25,7%	61,1%	8,9%	2,0%	6,8%	2,7%	6,7%	2,5%	61,3%	4,8%	12,8%	7,0%	16,2%	7,4%	59,8%	25,7%	10,4%	32,8%
EU071603 (*Staphylococcus capitis*)	5,8%	15,9%	42,8%	25,8%	51,8%	9,8%	7,2%	14,3%	11,8%	7,9%	15,3%	62,5%	12,6%	11,6%	20,3%	29,1%	0,0%	29,2%	41,2%	15,3%	18,6%
EU071623 (*Staphylococcus hominis*)	4,0%	6,6%	1,8%	6,8%	5,3%	64,3%	94,4%	13,2%	46,8%	10,3%	7,1%	9,5%	6,9%	9,5%	5,7%	15,6%	0,0%	50,7%	28,4%	10,1%	31,6%
EU071554 (*Bacillus licheniformis*)	92,0%	1,7%	4,1%	0,0%	100,0%	4,4%	0,1%	1,0%	4,9%	3,0%	5,4%	90,7%	2,5%	2,5%	4,3%	6,0%	0,6%	4,5%	77,2%	61,4%	49,9%
EU071584 (*Bacillus subtilis*)	1,1%	9,1%	15,6%	4,1%	25,4%	14,4%	1,8%	36,1%	9,3%	4,9%	6,2%	1,9%	2,6%	1,9%	3,4%	7,5%	0,6%	12,5%	100,0%	20,9%	95,7%
EU071614 (*Staphylococcus epidermidis*)	9,2%	7,0%	45,8%	68,0%	0,0%	10,8%	2,1%	14,7%	3,8%	29,3%	9,5%	77,9%	10,8%	7,3%	6,6%	10,4%	2,4%	46,0%	54,8%	9,9%	64,4%
EU071561 (*Bacillus pumilus*)	0,8%	1,8%	16,7%	0,0%	67,1%	3,3%	6,4%	68,8%	8,7%	6,4%	5,0%	65,0%	4,0%	3,5%	7,2%	0,0%	0,9%	2,5%	96,1%	4,7%	22,7%
EU071585 (*Bacillus subtilis*)	100,0%	1,2%	0,4%	0,0%	100,0%	4,5%	2,6%	9,9%	7,1%	3,9%	3,2%	1,7%	2,8%	2,2%	4,6%	0,0%	0,9%	8,8%	100,0%	6,8%	80,1%
EU071586 (*Bacillus subtilis*)	100,0%	10,4%	7,8%	13,1%	100,0%	5,5%	10,7%	5,7%	10,1%	5,1%	0,0%	39,2%	3,6%	5,0%	7,8%	16,6%	1,3%	8,3%	63,1%	5,0%	1,7%
EU071605 (*Staphylococcus epidermidis*)	7,0%	22,6%	33,4%	27,0%	61,3%	10,5%	7,9%	20,2%	10,8%	13,7%	16,5%	65,5%	7,7%	9,9%	13,2%	15,7%	0,7%	49,8%	44,8%	35,4%	2,2%
EU071547 (*Bacillus atrophaeus*)	84,8%	4,0%	8,1%	0,5%	91,2%	4,4%	4,8%	32,7%	6,0%	7,5%	7,5%	76,9%	5,3%	4,5%	4,8%	12,5%	1,2%	0,0%	91,7%	96,8%	64,6%
EU071601 (*Roseomonas mucosa*)	1,3%	17,0%	41,3%	6,1%	79,3%	7,8%	5,9%	57,2%	11,1%	6,4%	11,1%	14,3%	9,5%	6,7%	6,9%	20,1%	1,4%	0,0%	100,0%	87,8%	43,8%
EU071555 (*Bacillus licheniformis*)	98,4%	2,2%	6,6%	0,0%	100,0%	6,4%	1,3%	2,0%	1,8%	3,5%	3,9%	51,6%	1,6%	2,5%	2,1%	7,0%	3,7%	2,6%	18,3%	63,5%	63,0%
EU071556 (*Bacillus licheniformis*)	84,6%	5,6%	22,9%	10,6%	100,0%	7,0%	6,5%	7,2%	8,0%	10,1%	5,4%	100,0%	5,2%	5,2%	7,1%	10,3%	26,4%	51,4%	93,1%	7,6%	57,6%
EU071598 (*Paenibacillus graminis*)	82,7%	34,3%	42,3%	21,0%	18,8%	16,4%	13,8%	15,3%	12,0%	23,5%	10,7%	66,8%	9,6%	9,6%	24,9%	26,8%	2,0%	0,0%	32,4%	26,8%	5,4%
EU071626 (*Staphylococcus hominis*)	5,9%	20,0%	57,5%	11,6%	4,2%	7,0%	0,0%	9,5%	0,0%	7,6%	8,2%	67,1%	6,7%	9,8%	18,4%	13,6%	6,5%	51,6%	84,5%	19,2%	4,4%

Table 24: Sample code to GenBank accession number
This Table is also stored on the Data-CD.

Cultivable bacterial Isolates			Uncultivable bacterial Isolates		
Sample-code	**Genbank**	**Closest species (16S rDNA)**	**Sample-code**	**Genbank**	***Closest species (16S rDNA)***
EHFS1_AC2Ha	EU071578	*Bacillus subtilis (T) DSM10*	EHFS1_S01a	EU071465	*Peptostreptococcus anaerobius (T) ATCC 27337*
EHFS1_AU1Ha	EU071616	*Staphylococcus haemolyticus (T)*	EHFS1_S01b	EU071466	*Gemella morbillorum (T)*
EHFS1_S01Ha	EU071562	*Bacillus subtilis (T) DSM10*	EHFS1_S01c	EU071467	*Mogibacterium neglectum (T) ATCC 700924*
EHFS1_S01Hb	EU071563	*Bacillus subtilis (T) DSM10*	EHFS1_S01d	EU071468	*Rothia dentocariosa (T) ATCC 17931*
EHFS1_S01Hc	EU071617	*Staphylococcus hominis (T)*	EHFS1_S01e	EU071469	*Veillonella atypica*
EHFS1_S01Hd	EU071591	*Micrococcus luteus (T) type strain: DSM 20030*	EHFS1_S01f	EU071470	*Oribacterium sinus (T) AIP 354.02*
EHFS1_S01He	EU071607	*Staphylococcus epidermidis (T) ATCC 14990T*	EHFS1_S01g	EU071471	*Streptococcus parasanguinis (T) ATCC 15912*
EHFS1_S02Ha	EU071602	*Staphylococcus aureus (T)*	EHFS1_S01h	EU071472	*Gemella sanguinis (T) 2045-94*
EHFS1_S02Hb	EU071564	*Bacillus subtilis (T) DSM10*	EHFS1_S02a	EU071473	*Nocardioides sp. JC2055*
EHFS1_S02Hc	EU071565	*Bacillus subtilis (T) DSM10*	EHFS1_S03a	EU071476	*Haemophilus parainfluenzae (T)*
EHFS1_S02Hd	EU071592	*Micrococcus luteus (T) type strain: DSM 20030*	EHFS1_S03b	EU071475	*Granulicatella adiacens (T) GIFU12706*
EHFS1_S02He	EU071600	*Pseudomonas stutzeri (T) CCUG 11256*	EHFS1_S03c	EU071474	*Actinomyces odontolyticus (T) NCTC 9935*
EHFS1_S03Ha	EU071618	*Staphylococcus hominis (T)*	EHFS1_S03d	EU071478	*Veillonella parvula (T) DSM 2008*
EHFS1_S03Hb	EU071566	*Bacillus subtilis (T) DSM10*	EHFS1_S03e	EU071479	*Sphingomonas melonis (T) PG-224*
EHFS1_S03Hc	EU071567	*Bacillus subtilis (T) DSM10*	EHFS1_S03f	EU071477	*Rothia aeria (T) GTC867*
EHFS1_S03Hd	EU071568	*Bacillus subtilis (T) DSM10*	EHFS1_S04a	EU071480	*Mycobacterium chitae (T)*
EHFS1_S03Ta	EU071596	*Micrococcus luteus (T) type strain: DSM 20030*	EHFS1_S04b	EU071481	*Stenotrophomonas maltophilia (T) LMG 958-T*
EHFS1_S03Tb	EU071579	*Bacillus subtilis (T) DSM10*	EHFS1_S05a	EU071484	*Sphingomonas mali (T) IFO 10550-T*
EHFS1_S04Ha	EU071593	*Micrococcus luteus (T) type strain: DSM 20030*	EHFS1_S05b	EU071483	*Leptolyngbya sp. PCC 7104 (T)*
EHFS1_S04Hb	EU071569	*Bacillus subtilis (T) DSM10*	EHFS1_S05c	EU071482	*Lactobacillus sp. (T) CCUG 28746*
EHFS1_S04Hc	EU071619	*Staphylococcus hominis (T)*	EHFS1_S06a	EU071485	*Cellulomonas gelida (T) DSM 20111T*
EHFS1_S05Ha	EU071570	*Bacillus subtilis (T) DSM10*	EHFS1_S06b	EU071486	*Rickettsia honei (T) RB*
EHFS1_S05Hb	EU071559	*Bacillus pumilus (T) DSMZ27*	EHFS1_S07a	EU071473	*Novosphingobium aromaticivorans (T) SMCC F199*
EHFS1_S05Hc	EU071548	*Bacillus flexus (T) IFO15715*	EHFS1_S07b	EU071490	*Streptococcus thermophilus (T) DSM 20617*
EHFS1_S05Hd	EU071549	*Bacillus flexus (T) IFO15715*	EHFS1_S07c	EU071487	*Bacillus subtilis (T) IAM 12118T*
EHFS1_S05He	EU071557	*Bacillus muralis (T) type strain: LMG 20238*	EHFS1_S07d	EU071489	*Alpha proteobacterium 34632*
EHFS1_S05Hf	EU071620	*Staphylococcus hominis (T)*	EHFS1_S08a	EU071491	*Acinetobacter lwoffii (T) DSM 2403*
EHFS1_S05Ta	EU071599	*Paenibacillus macerans (T) ATCC 8244 and DSM 24*	EHFS1_S08b	EU071492	*Pseudomonas pseudoalcaligenes (T) LMG 1225T*
EHFS1_S05Tb	EU071551	*Bacillus licheniformis (T) DSM 13*	EHFS1_S09a	EU071493	*Bacillus benzoevorans (T) DSM5391*
EHFS1_S05Tc	EU071552	*Bacillus licheniformis (T) DSM 13*	EHFS1_S09b	EU071494	*Flavimonas oryzihabitans*
EHFS1_S05Td	EU071553	*Bacillus licheniformis (T) DSM 13*	EHFS1_S09c	EU071497	*Streptococcus australis (T) ATCC 700641*
EHFS1_S06Ha	EU071571	*Bacillus subtilis (T) DSM10*	EHFS1_S09d	EU071495	*Staphylococcus aureus (T)*
EHFS1_S06Hb	EU071604	*Staphylococcus epidermidis (T)*	EHFS1_S09e	EU071496	*Staphylococcus haemolyticus (T) ATCC 29970T*
EHFS1_S06Hc	EU071608	*Staphylococcus epidermidis (T) ATCC 14990T*	EHFS1_S10a	EU071503	*Actinomycete Lp2*
EHFS1_S06Ta	EU071580	*Bacillus subtilis (T) DSM10*	EHFS1_S10b	EU071500	*Knoellia subterranea (T) DSM 12332*
EHFS1_S07Ha	EU071572	*Bacillus subtilis (T) DSM10*	EHFS1_S10c	EU071502	*Pseudomonas rhizosphaerae (T) IH5*
EHFS1_S08Ha	EU071621	*Staphylococcus hominis (T)*	EHFS1_S10d	EU071504	*Variovorax paradoxus (T) DSM 66*
EHFS1_S08Ta	EU071581	*Bacillus subtilis (T) DSM10*	EHFS1_S10e	EU071501	*Streptococcus cremoris*
EHFS1_S09Ha	EU071573	*Bacillus subtilis (T) DSM10*	EHFS1_S10f	EU071499	*Knoellia sinensis (T) DSM 12331*
EHFS1_S10Ha	EU071574	*Bacillus subtilis (T) DSM10*	EHFS1_S10g	EU071498	*Corynebacterium tuberculostearicum (T) CIP107291*
EHFS1_S11Ha	EU071545	*Bacillus atrophaeus (T) JCM9070*	EHFS1_S11a	EU071505	*Catenibacterium mitsuokai (T) JCM 10609*
EHFS1_S11Hb	EU071546	*Bacillus atrophaeus (T) JCM9070*	EHFS1_S11b	EU071506	*Deinococcus radiopugnans (T) ATCC 19172T*
EHFS1_S11Hc	EU071594	*Micrococcus luteus (T) type strain: DSM 20030*	EHFS1_S11c	EU071507	*Hymenobacter roseosalivarius (T) AA718*
EHFS1_S11Ta	EU071582	*Bacillus subtilis (T) DSM10*	EHFS1_S11d	EU071508	*Staphylococcus warneri (T)*
EHFS1_S12Ha	EU071609	*Staphylococcus epidermidis (T) ATCC 14990T*	EHFS1_S12a	EU071511	*Haemophilus paraphrohaemolyticus (T)*
EHFS1_S12Hb	EU071575	*Bacillus subtilis (T) DSM10*	EHFS1_S12b	EU071510	*Clostridium lituseburense (T)*
EHFS1_S12Hc	EU071589	*Corynebacterium flavescens (T) NCDO 1320*	EHFS1_S12c	EU071509	*Bifidobacterium ruminantium (T) JCM8222*
EHFS1_S12Hd	EU071615	*Staphylococcus haemolyticus (T)*	EHFS1_S12d	EU071514	*Gram-positive bacterium MOL361*
EHFS1_S13Ta	EU071583	*Bacillus subtilis (T) DSM10*	EHFS1_S12e	EU071512	*Paracoccus carotinifaciens (T) E-396*
EHFS1_S14Ha	EU071560	*Bacillus pumilus (T) DSMZ27*	EHFS1_S12f	EU071513	*Paracoccus haeundaensis (T) BC74171*
EHFS1_S16Ha	EU071610	*Staphylococcus epidermidis (T) ATCC 14990T*	EHFS1_S13a	EU071518	*Micrococcus lylae (T) DSM 20315*
EHFS1_S17Ha	EU071550	*Bacillus licheniformis (T) DSM 13*	EHFS1_S13b	EU071517	*Micrococcus psychrophilum*
EHFS1_S17Hb	EU071611	*Staphylococcus epidermidis (T) ATCC 14990T*	EHFS1_S13c	EU071515	*Corynebacterium kroppenstedtii (T) CCUG 35717*
EHFS1_S17Hc	EU071576	*Bacillus subtilis (T) DSM10*	EHFS1_S13d	EU071519	*Staphylococcus caprae (T) ATCC 35538T*
EHFS1_S18Ha	EU071590	*Micrococcus luteus (T) ATCC 4698*	EHFS1_S13e	EU071520	*Streptococcus mitis (T) ATCC 49456*
EHFS1_S18Hb	EU071595	*Micrococcus luteus (T) type strain: DSM 20030*	EHFS1_S13f	EU071521	*Streptococcus sanguinis (T) ATCC 10556*
EHFS1_S18Hc	EU071577	*Bacillus subtilis (T) DSM10*	EHFS1_S13g	EU071516	*Gemella haemolysans (T)*
EHFS1_SConCCHa	EU071587	*Bacillus subtilis (T) DSM10*	EHFS1_S14a	EU071526	*Prevotella denticola (T)*
EHFS2_AC1Ha	EU071612	*Staphylococcus epidermidis (T) ATCC 14990T*	EHFS1_S14b	EU071524	*Neisseria canis (T)*
EHFS2_AC2Ha	EU071613	*Staphylococcus epidermidis (T) ATCC 14990T*	EHFS1_S14c	EU071523	*Beta proteobacterium B8*
EHFS2_AC2Hb	EU071625	*Staphylococcus hominis (T)*	EHFS1_S14d	EU071525	*Neisseria polysaccharea (T)*
EHFS2_AU1Ha	EU071622	*Staphylococcus hominis (T)*	EHFS1_S14e	EU071522	*Acinetobacter junii (T) DSM 6964*
EHFS2_AU1Hb	EU071606	*Staphylococcus epidermidis (T)*	EHFS1_S15a	EU071527	*Thermomonas brevis (T) type strain: LMG 21746*
EHFS2_AU1Hc	EU071603	*Staphylococcus capitis (T)*	EHFS1_S15b	EU071529	*Corynebacterium coyleae (T) DSM 44184*
EHFS2_AU1Hd	EU071623	*Staphylococcus hominis (T)*	EHFS1_S15c	EU071530	*Staphylococcus hominis (T) DSM 20328*
EHFS2_AU1He	EU071624	*Staphylococcus hominis (T)*	EHFS1_S15d	EU071528	*Burkholderia fungorum (T) LMG 16225*
EHFS2_S01Ha	EU071554	*Bacillus licheniformis (T) DSM 13*	EHFS1_S16a	EU071532	*Bacillus bataviensis (T) type strain: LMG 21833*
EHFS2_S01Ta	EU071584	*Bacillus subtilis (T) DSM10*	EHFS1_S16b	EU071533	*Clostridium disporicum (T) DSM 5521*
EHFS2_S01Tb	EU071614	*Staphylococcus epidermidis (T) ATCC 14990T*	EHFS1_S16c	EU071531	*Streptococcus defectivus*
EHFS2_S01Tc	EU071561	*Bacillus pumilus (T) DSMZ27*	EHFS1_S16d	EU071535	*Staphylococcus saccharolyticus (T)*
EHFS2_S01Td	EU071585	*Bacillus subtilis (T) DSM10*	EHFS1_S16e	EU071534	*Staphylococcus epidermidis (T) ATCC 14990T*
EHFS2_S01Te	EU071586	*Bacillus subtilis (T) DSM10*	EHFS1_S16f	EU071536	*Streptococcus sinensis (T) HKU4*
EHFS2_S03Ha	EU071605	*Staphylococcus epidermidis (T)*	EHFS1_S17a	EU071538	*Amaricoccus macauensis (T) Ben104*
EHFS2_S04Ha	EU071547	*Bacillus atrophaeus (T) JCM9070*	EHFS1_S17b	EU071540	*Methylobacterium fujisawaense (T) DSM 5686*
EHFS2_S05Ha	EU071558	*Bacillus muralis (T) type strain: LMG 20238*	EHFS1_S17c	EU071537	*Acinetobacter johnsonii (T) DSM 6963*
EHFS2_S05Hb	EU071601	*Roseomonas mucosa (T) MDA5527 ATCC BAA-692*	EHFS1_S17d	EU071541	*Streptococcus pneumoniae (T) ATCC 33400*
EHFS2_S05Hc	EU071588	*Bacillus vallismortis (T) DSM11031*	EHFS1_S17e	EU071539	*Megasphaera micronuciformis (T) AIP 412.00 CIP 107280*
EHFS2_S05Hd	EU071555	*Bacillus licheniformis (T) DSM 13*	EHFS1_S18a	EU071543	*Fusobacterium canifelinum (T) RMA 12708 Q11*
EHFS2_S05Ta	EU071556	*Bacillus licheniformis (T) DSM 13*	EHFS1_S18b	EU071544	*Staphylococcus cohnii (T) ATCC 49330T*
EHFS2_S05Tb	EU071597	*Paenibacillus graminis (T) RSA19*	EHFS1_S18c	EU071542	*Filifactor alocis (T) ATCC 35896*
EHFS2_S05Tc	EU071598	*Paenibacillus graminis (T) RSA19*			
EHFS2_S08Ta	EU071626	*Staphylococcus hominis (T)*			

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1979 - 1983	Elementary school, Bonn-Bad Godesberg, Germany
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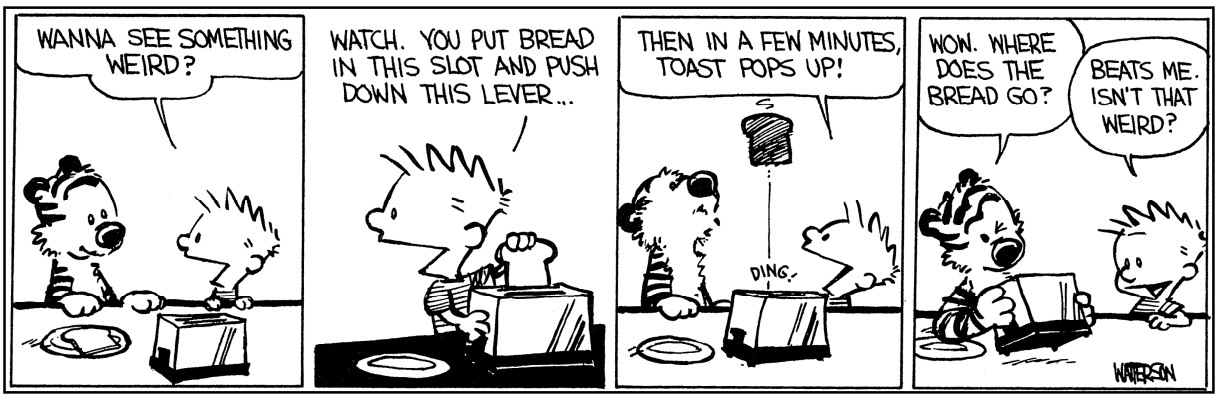
1993	Abitur
1993	Matriculation at the University of Bonn, Germany Field of study: Biology
1995	Intermediate diploma examinations (botany, cell biology, genetics, zoology and physics)
1998	Master of arts (genetics), University of Kansas, USA
1999	Diploma examinations (cell biology, genetics and physics)
2000	Experimental diploma thesis at the Institute for Animal Anatomy and Physiology, Department of Biochemistry, PD. Dr. R. Probstmeier, University of Bonn, Germany Title of the Diploma thesis: Regulation and expression of galectin-3 and tenascin-R during peripheral nervous system development
2000 - 2002	Research associate at the Department of Immunology, Prof. Dr. N. Koch, University of Bonn, Germany
2003 - 2006	Experimental work for this doctoral thesis at the Institute of Aerospace Medicine, Radiation Biology Division, Research Group Photo- and Exobiology, German Aerospace Center (DLR), Germany



There always will be mysteries that need to be solved…