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PART A: PROPOSED FUTURE WORK FOR GENETIC STABILITY STUDY

prepared by/*préparé par*	L. Hendrickx, R. Wattiez, S. Baatout, A. Wilmotte, P. Janssen, M. Mergeay
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1 INTRODUCTION

The effect of standard stress conditions was tested on *R. rubrum* ATCC25903 and *Arthrospira* sp. PCC8005 at the cellular, proteomic and genetic level. Several techniques were investigated on strains for compartment 1, 2, 4a and the most promising ones were set-up and validated to study the response of relevant strains (*R. rubrum* ATCC 2590, *A. platensis* PCC 8005, R. *metallidurans* AE128) to environmental stress at different cellular levels (e.g. AFLP fingerprinting at genetic level, 2D-gel & MALDI-TOF-MS fingerprinting at proteomic level and flow cytometry of dyed cells at cellular level). It should be noted that these techniques were firstly selected because of their applicability for studying non-sequenced strains as *A. platensis* and *R. rubrum* at the stage of Phase 1.

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2 MELGEN, THE TOOLS

2.1 *Measuring metabolic stability and stress response using flow cytometry*

Certain morphological changes have been reported in the literature following temperature, oxidative or pH stress. These include compression of gas vacuoles, cell elongation or shortening, separation of the membrane from the cell wall, pore formation, alterations of the cytoskeleton, nucleus, and cell organelles, coagulation of plasma proteins and release of cell constituents to the exterior. Temperature, H_2O_2 or pH treatment is also known to induce alterations in the composition of the cell membrane, with resultant phase transitions from liquid into gel.

In phase 1 of MELGEN, we were interested in the application of flow cytometry to physiological studies of some of the Melissa bacterial strains following stresses that can happen in space. The results presented in MELGEN, phase 1, indicated that the *R. metallidurans*, *R. rubrum* and *Arthrospira* sp. strains showed different staining behaviours with a series of various fluorochromes meaning that physiological characterisation of the strains reveals a difference in the resistance of the strains to oxidative, temperature or pH stress. *R. rubrum* seemed to show higher sensitivity to temperature and oxidative stresses than *R. metallidurans*. Concerning *Arthrospira* sp., small and big filaments did not react the same way and temperature or oxidative treatment yielded varying results. In all three strains, a correlation was observed between membrane integrity and potential. Following oxidative stress, the membrane permeability and potential increased in function of the concentration of H_2O_2.

In conclusion, flow cytometry has been shown to be a fast means of obtaining information about physiological status and metabolism of the MELiSSA strains that provides individual cell information. It makes it an ideal tool not only to understand the influence of various stresses (for example, X and UV-irradiations, pressure, starvation or physical stress) on homogeneous (or heterogeneous) populations but also to be used in routine controls of the strains present in the MELiSSA bioreactors. Finally, flow cytometry with specific fluorescent probes, for example specific mRNA probes, could be used to follow changes in gene expression patterns that are consequent to stresses.

2.2 *Measuring metabolic stability and stress response using 2D-Proteomics*

A proteomic approach based on two-dimensional gel separation and mass spectrometry protein identification was used to detect and identify protein modifications in different stress conditions (temperature, pH variation and oxidative stress).

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First, 2D maps of *C. metallidurans* were build to validate the method of 2D proteomic mapping as a method to detect stress. In this study, we constructed a database of total cellular proteins from *Ralstonia metallidurans* CH34. This database will serve in the future as a reference to which proteins differentially expressed, consecutively to exposure to a variety of stress and environmental conditions (i.e. space conditions), will be compared. Protein identification was carried out via N-terminal amino acid sequencing, MALDI-TOF MS and tandem MS. So far 1400 different proteins were characterized out of 1600 protein spots. 99.9 % of proteins tested with the MALDI-TOF MS have been identified with success. Although the proteome map is still not complete, one could appraise the importance of proteomics for genome analyses (especially by the MALDI-TOF approach) through (1) the identification of previously undetected open reading frames, (2) the identification of proteins not encoded by the already sequenced genome fragments, (3) the characterization of protein-encoding genes spanning two different contigs, enabling their merging, and (4) the precise delineation of the amino-terminus of several proteins. Finally, this map proved a useful tool, firstly, in the identification of proteins differentially expressed in the presence of low amounts of different heavy metals and secondly, in the identification of the mechanism to use acetone as carbon source in *R. metallidurans*. These last results are the first demonstration of the ability of *R. metallidurans* to use the acetone as carbon source.

This approach and technology, particularly appropriate to begin a proteomic study from uncompleted bacterial genome will be used to detect and identify protein modifications in different stress conditions (temperature variation, oxidative stress, space conditions...) especially with the MELiSSA strains as *Arthrospira* sp. PCC8005 and *Rhodospirillum rubrum* ATCC25903.

With the DNA sequences of more genomes being completed, the sequence of *Ralstonia metallidurans* CH34 and *Rhodospirillum rubrum* genomes, a major challenge in modern biology remains the understanding of the expression, function, and regulation of the entire set of proteins encoded by a micro organism: proteomics. This information will be valuable for understanding how complex biological processes occur at the molecular level, how they differ in various microorganisms, and how they are altered in different growing conditions as space conditions particularly in the case of the MELiSSA project. The spectrum of proteins expressed in a microorganism provides that cell with a unique identity, elucidating how the protein complement changes in a cell type during development in response to environmental stimuli as oxidative stress, temperature or pH variation. Recent years have witnessed a revolution in the development of new approaches for identifying large numbers of proteins expressed in cells and also for globally detecting the differences in levels of proteins in different cell states. In recent years, protein separation methods as the two-dimensional gel electrophoresis coupled with various mass spectrometry (MS) technologies have evolved as the dominant tools in the field of protein identification and protein complex deconvolution. This approach is probably the best approach to characterise the proteome especially if the genome is partial or unknown. Among the different steps of this approach, the protein extraction is crucial for the outcome of this approach.

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In this context, an extraction protocol compatible with a proteomic approach using the two-dimensional gels electrophoresis separation was initiated for the two MELiSSA strains: *Rhodospirullum rubrum* and *Arthrospira* sp.. This approach should allow as it already has successfully been used for *C. metallidurans* to study the up- or down regulated proteins in bacteria growing in different environmental conditions as oxidative stress, temperature variation, space conditio by a differential comparison. The preliminary results showed already more known proteins up-regulated during temperature variations (GroEs, GroEL, Dnak and HtpG) and oxidative stress (GroES, Thioredoxine, RecA and Alkyl hydroperoxyde reductase subunit C). For *Rhodospirillum rubrum* ATCC25903 a number of stress-induced proteins were already identified. Under conditions of thermic stress, the chaperone proteins GroEL, GroES, DnaK and HtpG were induced. Under oxidative stress the induced proteins were identified as thioredoxin, alkylhydroperoxyde reductase, HtpG and RecA. Thioredoxin and alkylhydroperoxyde reductase are proteins known to be induced under oxidative stress. HtpG on the other hand is a typical heat shock protein, and RecA is involved with gene repair. Unexpectedly there was no induction of superoxide dismutase observed. To obtain an induction of superoxide dismutase it was probably necessary to increase the concentration of H_2O_2 or the time of incubation. The induction of HtpG during oxidative or thermic stress has already been observed in the literature and indicates that certain proteins can be induced as a general response against stress.

In conclusion, the proteomic approach based on 2-DE coupled to mass spectrometry has shown to be an excellent means of obtaining information about physiological status and metabolism of the MELiSSA strains that is almost impossible to obtain in any other way, especially with an non or partially characterise genome. It makes it an ideal tool to understand at the molecular level the influence of various stresses (for example, X and UV-irradiations, pressure, starvation, pH stress) on the strains present in the MELiSSA bioreactors. Finally, it is important to note that the proteomic approach could be used to understand the behaviour of the MELiSSA bioreactors in different growth conditions.

2.3 Measuring metabolic stability and stress response using MALDI-TOF-MS

In the context of the MELiSSA loop, temperature, pH and oxidative stresses are a perplexing risk for *Rhodospirillum rubrum* ATCC25903 and *Arthrospira* sp. PCC8005 and understanding the physiological mechanisms induced by those stresses. In phase 1 of MELGEN we reported the success of mass spectrometry as a powerful technique to monitor with a high degree of statistical resolution, cell modifications during temperature, oxidative ad pH stress of *R. rubrum* and *A. platensis*. Moreover, these results confirmed identical observations obtained by another approach based to the flowcytometry analysis.

A variety of characteristics of microorganisms (both phenotypic and genotypic) are used to differentiate between strains and often to determine the relationship between strains of bacteria present on the bacterial cell wall are surface components, which give rise to a unique pattern of

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biomarkers. These surface components are important since they mediate the contact between the cell and the environment. This unique population of molecules can be rapidly desorbed from the cell surface; ionised and analysed by matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF-MS) resulting in a mass fingerprint. This approach should be rapid and yet be based on a sufficiently large group of proteins so that a unique mass spectral fingerprint for the organism or strain can be obtained. Some of the difficulties involved in the MALDI-TOF mass spectral analysis of bacteria have been the complexity of the spectra, the large mass ranges used, and the subtle differences that may be observed in spectra from related stains. Moreover, reproducibility is a difficult problem in experiments that involve MALDI of cells, and large variations have been seen in spectra obtained under different conditions. There are many experimental parameters that can have a strong effect on the observed mass spectra (minor variations in the sample/matrix preparation, in the experimental conditions used to bacterial extraction or analysis). In MELGEN, phase 1 we reported on the success of the intact mass spectrometry to study statistically and with a high reproducibility *Rhodospirillum rubrum* ATCC25903 and *Arthrospira* sp. PCC8005.

In phase 1, the following stresses were detected: temperature, oxidative and pH stresses. Another initially perplexing issue was the time dependence of spectra noted upon analysis of bacteria from cultures. Bacteria respond rapidly to environmental changes, and the production of stress proteins or other similar changes in cellular processes results to a modification in the spectra for reasons associated with the biology of bacteria. Certain morphological changes have been reported in the literature following temperature, pH or oxidative stress. These include compression of gas vacuoles, cell elongation or shortening, separation of the membrane from the cell wall, pore formation, alterations of the cytoskeleton, nucleus, and cell organelles, coagulation of plasma proteins and release of cell constituents to the exterior. Temperature and pH treatment is also known to induce alterations in the composition of the cell membrane, with resultant phase transitions from liquid into gel.

Interestingly, pigments and proteins from chlorosomes, the light-harvesting organelles from the photosynthetic bacterium could be characterized directly from organelles. Recent work shown that by applying a small volume of a concentrated suspension of isolated chlorosome organelles directly onto the MALDI target, bacteriochlorophyll a and the major homologs of bacteriochlorophyll c have been characterised from *Chlorobium tepidum.* Interestingly, the authors noted that the peak surface of the different bacteriochlorophyl in the MALDI spectra were proportional to peak areas obtained by HPLC analysis. Similar results were reported when whole cells were applied to the target. The MALDI-TOF can rapidly provide semiquantitative analysis as well as a fingerprint for the small amount pigments present in bacteria. This approach could be applied to the MELiSSA strains to monitor their light-harvesting systems in different growth conditions.

Our results demonstrated that MALDI-TOF of intact cells could be used to monitor biological changes, such as those that occur during oxidative, temperauture and pH stress. This whole-cell approach to the monitoring of biological changes would, of course, only allow a fraction of the environmental-response related proteins to be detected, compared to similar studies with

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isolated or fractionated protein samples, but the rapidity of the analysis often offsets this limitation. Moreover, our results indicate that the *R. rubrum* and *Arthrospira* sp. strains showed different behaviours to oxidative, pH and temperature stresses. *R. rubrum* seemed to show higher sensitivity to temperature, pH and oxidative stresses than *Arthrospira* sp. Interestingly, these results were in good concordance with those obtained by flow cytometry. Moreover, in both strains, a correlation was observed between membrane integrity and the mass spectrum modifications observed during stress variations.

Nevertheless, the MALDI-TOF-MS approach is not able, without a prefractionation, to monitor specific physiological modifications from heterogeneous populations of a bacterial strain such as *Arthrospira* sp., which is constituted of small and big filaments.

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3 FUTURE DIRECTIONS TO ASSES GENOMIC/METABOLIC STABILITY

3.1 Effect of space related stress conditions on R. rubrum

3.1.1 INTRODUCTION

Because long haul space exploration mission cannot be considered without a high-efficient and, above all, high-reliable biological life support system, the global behaviour of the support organisms in space conditions need an in depth investigation. Therefore, the general objective is to investigate the effect of spaceflight related environmental conditions on the MELiSSA loop's phototrophic bacteria *R. rubrum* ATCC25903. To complete this goal, we will consider *R. rubrum*'s response to:

(1) Ionizing radiations and oxidative stress
(2) Long-term culturing

At the end of our work, we will be able to establish a global and complementary study on the proteomic, transcriptomic and genotypic responses of a phototrophic bacterium to radiation conditions. Furthermore, this study will enhance our comprehension of evolutionary parameters related to long time culturing of phototrophic microorganisms.

3.1.2 THE STRATEGY OF THE 'GLOBAL APPROACH'

(a) Total metabolic bacterial response on proteomic level

The analysis of the proteome involves the resolution of the proteins in a sample followed by the identification of the resolved proteins. 2D-**PAGE** (**P**oly**A**crylamide **G**el **E**lectrophoresis) followed by **M**ass **S**pectrometry (**MS**) is the most widely used method of protein resolution and identification (Herbert *et al.*, 2001; Lopez and Pluskal, 2003). In 2D-PAGE, proteins are separated in one dimension by isoelectric point and in the other dimension by molecular weight. As a result, a single 2D-PAGE system (2D-map) can resolve more than 4000 proteins (Celis and Gromov, 1999). This allows differential expression studies at the protein level by comparison between two different 2D-maps (Noël-Georis *et al.* 2004). Identification of the proteins is then achieved by using **M**atrix-**A**ssisted **L**aser **D**esorption **I**onisation (**MALDI**)-**T**ime **o**f **F**light (**TOF**) MS. This approach has already been successfully used with the bacteria *Ralstonia metallidurans* CH34 showing an up-and-down protein regulation in relation with spaceflight conditions (Wattiez, personal communication).

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Intact **C**ell **M**ALDI-TOF **M**ass **S**pectrometry (**ICM-MS**) combined with novel bioinformatic approaches provides a powerful new strategy for the rapid speciation and typing of microorganisms (Hillenkamp *et al.*, 1991). This method allows the rapid sampling of a population of macromolecules expressed on the surface of bacteria and characterization of these molecules by molecular weight (Evason *et al.*, 2001; Walker *et al.*, 2002). The resulted mass spectrum provides a unique physico-chemical fingerprint for the species tested.

(b) Total metabolic bacterial response on transcriptomic RNA level

The expression of genes in bacteria is regulated by environmental conditions. Hence variations in environmental stimuli will result in differential expression of the genes. This transcriptomic level of metabolic bacterial response will be investigated using miniaturized DNA hybridisation systems: DNA chips (or DNA microarrays).

A DNA microarray is basically a glass slide where the genes of an organism can be spotted. Each gene is probed with fluorescently labelled mRNA obtained from the organism of interest grown under a specific condition. Locations where the RNA has hybridised to the DNA is indicated by a gradation of colour up to maximum hybridisation. Because the location of different genes on the chip is known, DNA-chip analysis will reveal which specific genes were expressed. This allows simultaneous detection of expression of thousands genes and reveal the manner in which an organism uses its genetic arsenal under various conditions (Stephens, 2001).

(c) Genetic stability and rearrangements

In nature, genetic diversity in prokaryotes is driven largely by a number of dynamic processes that enable them to react swiftly to changes in their environment. To accomplish this "adapt-to-survive" strategy, microbes have many routes at their disposal to acquire beneficial, or eliminate superfluous, genetic material, and to "reshuffle" genes that need to be expressed at short notice (Casjens, 1998; Arber, 2000).

These genetic rearrangements will be analysed by **AFLP** (**A**mplified **F**ragment **L**ength **P**olymorphism) fingerprinting. AFLP can be largely divided into three steps (Vos *et al.*, 1995): (1) digestion of total cellular DNA with two restriction enzymes and ligation of restriction half site-specific adaptors to all restrictions fragments; (2) selective amplification of some of these fragments with two **PCR** (**P**olymerase **C**hain **R**eaction) primers that have corresponding adaptor and restriction site sequences; and (3) electrophoretic separation of amplicons on a gel matrix followed by visualisation of the banding pattern.

The AFLP method has great flexibility in that many primer pairs may be used on the same template. This means that a large numbers of nucleotides distributed over the entire genome can be surveyed simultaneously. This approach allows the detection of rare polymorphisms, is particularly interesting for the analysis of highly related genomes and, owing to its great

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resolution, is perfectly suited to follow the genetic stability of prokaryotic organisms (Janssen *et al.*, 1996).

3.2 Effect of radiation and oxidative stress on R. rubrum ATCC25903

3.2.1 EFFECT OF RADIATION ON A CELL

These effects will be dependent on the dose, the radiation type, the dose rate, the cellular type and the physiological state of the cell. Radiations may provoke injuries to biomolecules (DNA, proteins, etc) but due to the natural occurrence of radiation in the environment (see below), cells have developed some reparation mechanisms (from Vuillez, 2003).

When a cell absorbs radiation, there are three possible effects on the cell:
(1) The cell cannot repair the damages. This will cause cell death;
(2) The cell performs an incorrect/incomplete reparation. This may involve cell death or a non lethal mutation;
(3) The cell recovers completely from the damages it had undergone.

3.2.2 RADIATION ENVIRONMENT

Astronauts, cosmonauts and taikonauts aboard a LEO spacecraft such as NASA Space Shuttle and ISS or on board spaceships travelling outside the Earth's magnetosphere on missions to and from the Moon or Mars are exposed to levels of radiation far in excess of those encountered on the ground. Moreover these radiation conditions are dependent on a large number of parameters including the altitude and inclination of the spaceship's orbit, the orientation of the spaceship relative to the Earth and Sun and the particular phase of the 11-year solar cycle (Benton and Benton, 2001).

(a) Radiation environment in LEO

Ionizing radiations

The three principal sources of ionizing radiations in LEO are the following: (1) **G**alactic **c**osmic **r**ays (**GCRs**) are charged particles (protons, α-particles and heavy ions) that originate from beyond the solar system; (2) Energetic electrons and protons are trapped in the geomagnetic field and make up the **E**arth's **r**adiation **b**elts (**ERBs**) and (3) **S**olar **p**article **e**vents (**SPEs**) are high fluxes of charged particles (mostly low-energy protons and α-particles) encountered during rare but intense solar flares and **C**oronal **m**ass **e**jections (**CMEs**) (Nicholson *et al.*, 2000; Benton and Benton, 2001).

When passing through the skin and structure of a spaceship, primary ionizing particles can undergo interactions with nuclei that constitute the spaceship's mass, producing a wide variety of secondary particles: neutrons, protons, recoil nuclei, projectile fragments, γ-particles, etc.

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While the number of different particle species is large and the energy spectrum they occupy quite broad, their fluxes are often low but relatively rare events associated with solar flares and CMEs can produce sudden and dramatic increases in flux. Spaceship shielding is thus one of the most important factors in determining the characteristics of the ionizing space radiation inside a spaceship (Benton and Benton, 2001).

Earlier measures on Mir Orbital Station taken during a period of eleven year (1986 to 1997) show dose rate ranging from 162 to 508 µGy/day (Benton and Benton, 2001). Recent monitoring of radiations from April 2002 to may 2003 onboard ISS gives an average of 157 µGy/day (Golightly, personal communication). Background radiation levels on Earth which come from a combination of terrestrial (from the ^{40}K, ^{232}Th, ^{226}Ra, etc.) and cosmic radiation (photons, electron, etc.), are fairly constant over the world, being 2-4 µGy/day (ISU, 2004). Hence, onboard ISS, organisms undergo radiation stress up to 40-fold higher than their usual terrestrial environment.

(b) Radiation environment beyond the magnetosphere

Ionizing radiation

The ionizing radiation environment outside the Earth's magnetosphere differs markedly from that encountered in LEO. Not only are the trapped radiation belts absent, but also interplanetary space lacks the protection afforded by the Earth's magnetic field and the associated geomagnetic cut-off. As an example, the Apollo measurements reflect the specific trajectory taken by the spacecraft through the ERB in transit to and from the Moon. Mean dose rate for the Apollo missions ranged from 220 to 1270 µGy/day (Benton and Benton, 2001). Thus, in deep space the ionizing radiations stress can reach up to 300-fold those related to the terrestrial environment.

(c) Radiation on Mars

Mars is regarded as the most interesting planet in our solar system in a search for life beyond the Earth. This is mainly based on the fact that the early histories of Mars and Earth show similarities during the period when life emerged on Earth.

Exposure to space radiation on the Martian surface is greatly reduced by the shielding provided by the planet itself. The thin, largely CO_2, atmosphere of Mars provides ~16 g/cm^2 of shielding (as compared to 1030 g/cm^2 provided by Earth's atmosphere at sea level). Oxygen is present only as trace element, in very low concentrations, therefore an UV-absorbing ozone layer cannot be formed as on Earth. Moreover, Mars lacks a strong magnetic field to deflect lower energy particles away. Thus, on the Martian surface, exposure to space radiations, while reduced compared to that encountered in free space, is nonetheless omnipresent (Benton and Benton, 2001). Today, our knowledge about the radiation conditions on the surface of Mars and their biological effectiveness is still based on radiation measurements by instruments residing in Mars' orbit (Horneck *et al.*, 2001; Saganti *et al.*, 2002). However, the recent landing

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of NASA's rovers 'Spirit' and 'Opportunity' (and maybe ESA's 'Mars Express') at the beginning of this year will certainly fill this gap.

Regarding ionizing radiation, Mars Odyssey Orbiter gives mean data (for the period from 03/13/2002 to 09/30/2003) ranging from 200-300 μGy/day to up to 20,000 μGy/day during solar particle events (NASA, 2004).

Table 1: Physical conditions prevailing on Earth, in LEO, in interplanetary space and on Mars (different sources, see text above).

Radiation type	Earth	LEO	Interplanetary space	Mars
UV wavelengh (nm) UV biological effective dose (J_{eff}/m^2)	>290 ~$6*10^{-2}$	>170[a] 63[a]	>170 ~63	>200 not available
Ionizing (μGy/day)	2-4	160-500[b]	220-1270	200-300

[a]: outside space station;
[b]: inside space station.

3.2.3 OXIDATIVE STRESS

Reactive forms of oxygen (O_2^-, H_2O_2 and OH^-), generated naturally by aerobic metabolism, induce severe cellular damages like protein, membrane and nucleotide alterations. Besides that natural occurrence of free radicals, oxidative stress can also be related to radiations stress. Indeed, in addition to direct effect on biomolecules, UV and ionizing radiations will cause direct degradation of water into free radicals. Because the concentration of these reactive forms must be kept low in order to stay compatible with cellular integrity, bacteria developed powerful protection systems involving enhanced production of radical scavenging proteins. As an example, cells of *Escherichia coli* and *Salmonella typhimurium* show highly increasing synthesis rate of 40 proteins after an H_2O_2 exposition (Lynch and Lin, 1996).

3.2.4 EXPERIMENTAL STRATEGY

(a) Effect of radiation stress using SCK·CEN radiation sources

As stated above, cells have mechanisms to repair the continuous damages provoked by natural occurring radiations. Therefore it is relevant to try different exposure and recuperation times in order to assess how the cell can recover from radiation stress.

The effects of radiation will be assessed using both high and low **LET** (**L**inear **E**nergy **T**ransfer) radiations: γ-rays, X-rays (low LET) and neutrons (high LET):

(1) Different exposure times;
(2) Different recuperation times after exposure;
(3) Using similar radiation doses as found inside the ISS (or Mir), and on Mars;

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(b) Effect of simulated cosmic radiation

It is safe to assume that the composition of the radiation beam will likewise have an effect on microorganisms in comparison with irradiation using single type beams. The composition of cosmic radiation is dramatically different from Earth's radiation environment. Therefore, an experiment will attempted to be conducted in a space radiation simulator, the **C**ERN-**EU** high energy **R**eference **F**ield (**CERF**) facility based in Switzerland. Its function is to provide a reference base for testing, intercomparing and calibrating passive and active instruments before their use on-board aircraft and in space (Mitaroff and Silari, 2002).

(c) Effect of oxidative stress

Oxidative stress is generated by addition of hydrogen peroxide (H_2O_2) at different concentrations to the bacteria suspension.

(d) Methods used to monitor the effects

The effects of the stress investigated will be monitored at different levels:
(1) Proteomic level by 2D analysis and ICM-MS (see *1.2.1. (a)*) (preliminary experiments already done for the oxidative stress - UMH, unpublished results);
(2) Transcriptomic RNA level by DNA chip analysis (see *1.2.1. (b)*);
(3) Genetic stability and rearrangement by AFLP after viability testing and/or phenotype selection (see *1.2.1. (c)*).

3.3 Effect of long-term culturing on R. rubrum ATCC25903

3.3.1 GENOMIC EVOLUTION

As we have seen above, genetic stability is of prime importance for biological life support system. Therefore, evolutionary aspects are needed to be assessed with the MELiSSA organisms.

Genetic diversity is a balanced interplay between mutation, isolation and natural selection. Genetic variation can be increased by three different strategies: local sequence change, DNA rearrangement and DNA acquisition. Local sequence change is caused by replication infidelity. Internal and environmental mutagens can likewise cause local sequence change as well as DNA rearrangement. Furthermore, DNA acquisition can be accomplished by horizontal gene transfer (Arber, 2000).

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3.3.2 EXPERIMENTAL STRATEGY

(a) Culture conditions

R. rubrum is a very versatile organism, it will be therefore desirable to test genomic evolutionary changes in several culture media. However, due to the limited amount of bioreactors present in the lab, only one continuous culturing experiment (chemostat) can be conducted in this project.

The following culture conditions will be investigated:
(1) Photoheterotrophic media will be tested in continuous culture during 1-2 years;
(2) Photoheterotrophic, photoautotrophic, chemoheterotrophic and chemoautotrophic media will be tested using the so-called Lenski experiment (batch grown cultures that are diluted at specific intervals) (Lenski and Travisano, 1994).

(b) Genomic evolution during long-term culturing

Total genome dynamics of the culture will be investigated:
(1) Genetic stability and rearrangements, assessed by AFLP (see *1.2.1. (c)*);
(2) Movement of **IS** (**I**nsertion **S**equences) element using PCR techniques.

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3.4 Construction, validation and use of the DNA chip for R. rubrum ATCC25903

3.4.1 INTRODUCTION

DNA Microarrays are a relatively new technology that allow researchers to monitor the RNA expression levels for thousands of genes at one time (DeRisi *et al.*, 1997). The underlying technique of hybridization to a known sequence has been used for quite some time in techniques such as Southern and Northern blots. DNA Microarrays have extended this basic technique by using much smaller amounts of DNA probe, and more importantly by allowing researchers to perform tens of thousands of hybridization experiments in parallel. This allows researchers to view the response of whole genomes to various stimuli.

3.4.2 DNA CHIPS, THEIR USE, THEIR PROBLEMS, THEIR BENEFITS

Experimental layout. The actual mechanics of making and using DNA microarrays is non-trivial. In order to construct microarrays with thousands of probes on a standard microscope slide it is necessary to place each spot only 200 microns away from it's nearest neighbor. To accomplish this high density robotics are used (Cheung *et al.*, 1999). In general a robot is used to spot many different DNA molecules, which will be the *probes* for the microarray onto pre-treated glass microscope slides. The DNA is thereby covalently bound to the slide (Fig. 6.1.). In our laboratory, spotting is performed using the MicroGrid system of BioRobotics.

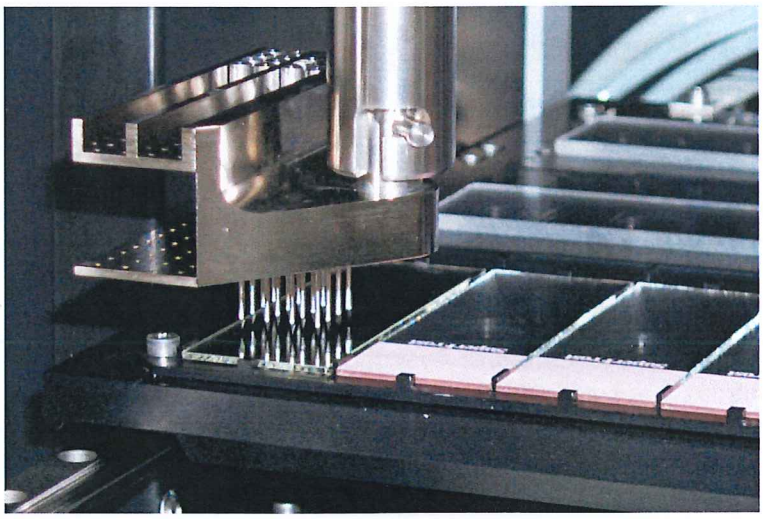

Fig. 6.1.: Robotic spotting of microdroplets containing DNA probes. Spots are less then 200 microns away from each other and up to 10,000 spots can be easily applied on one single glass slide.

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Once the microarray has been constructed it can be used for hybridization. The general protocol for performing hybridizations is as follows (Fig. 6.2.):
(1) RNA is extracted from an experimental state of interest, e.g. a bacterial strain exposed to some form of stress. RNA is also extracted from a control or *reference* state, e.g. a bacterial strain grown under normal conditions.
(2) The RNA from each state is reverse transcribed to form a cDNA from the original RNA, this is referred to as the *target*. The cDNA from the experimental and the reference state are labeled with different fluorescent dyes. i.e. Cy3 and Cy5.
(3) The cDNA targets are mixed together and are hybridized to the microarray. After hybridization the microarray is washed to remove non-specific binding.
(4) The microarray is then scanned using essentially a modified fluorescent microscope with a photomulitplier tube attached. This translates the intensity of each fluor hybridized to the microarray into separate tiff (tagged image format) files.
(5) The pixel intensities in the resulting tiff files are then compared to determine changes of RNA quantity (ergo, gene expression) in the experimental vs. the control states.

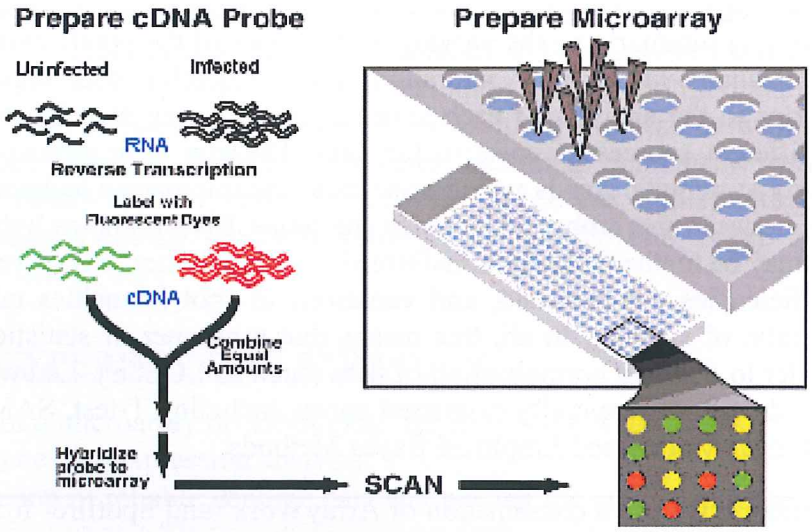

Fig. 6.2.: Experimental design of a microarray

Probe design. One approach to make probes for microarrays is to use PCR to amplify and isolate gene-specific sequences and spot these amplicons on glass slides. However, this involves the amplification of perhaps thousands of gene sequences in which primer design and prevention of cross-hybridisation are often cumbersome. As an alternative, it has now become possible both financially and chemically to use instead oligonucleotides as probes and bind them covently to glass supports. Currently, 50- to 70-mer probes are favoured.
Although such oligonucleotides offer greater technical and functional benefits it is first necessary to design probes that can fulfill these objectives. While amplicon probes are imprecise they do contain a lot of sequence. Oligonucleotides probes are much smaller and it is

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important to avoid sequences that will be complementary to more than one gene, that will have secondary structure, and sequences with binding energies that are too high or too low. Ideally, the hybridisation properties of oligonucleotide probes spotted on a single array should be alike. This means for instance that the melting temperature T(m) of all probes should be in the same range implying a limited variation in length and sequence.

The software we use to design 50-mer probes is OligoArra v2.0 (Rouillard *et al.*, 2003). This program allows the identification and design of oligonucleotide probes at the genomic scale using a thermodynamic approach to predict secondary structures and to calculate the specificity of targets on chips for a unique probe in a mixture of labeled probes. OligoArray 2.0 can also adjust the oligonucleotide length - according to user input - to fit a narrow T(m) range compatible with user-qualified hybridization requirements and checks for possibly cross-hybridisation using BLAST (Best Local Allignment Search Tool) (McGinnis & Madden, 2004). The OligoArra v2.0 software is freely available[1].

Data analysis pipeline. There are a number of important steps in microarray analysis. First, image quantification is important. This includes: (i) laying the grid and finding the printed spots in the image, (ii) identifying the extent of each spot, and separating foreground from background, (iii) summarizing the varying brightnesses of the pixels in the foreground of each spot , (iv) dealing with scanner saturation, and (v) dealing with variable backgrounds. In addition, prior to the analysis of each slide, a preprocessing step has to be performed since there are different sources of noise in the data. The aim is to remove from the expression measures any systematic trends which arise from the microarray technology rather than from differences between the probes or between the target RNA samples hybridized to the arrays. Thus, data may be mathematically transformed, missing values may be replaced, differences in labeling efficiencies compensated, and variations in spot intensities taken into account (by taking the ratio of signals). In all, this means that a number of statistical measures must be taken in order to obtain a normalisation of data (such as LOESS). Likewise, statistical test are required to identify differentially expressed genes, including T-test, SAM, and ANOVA, and the use of Linear Models and Empirical Bayes Methods.

In our laboratory, we use a combination of ArrayWorx[2] and Spotfire[3] for image quantification and processing. For normalisation and further data analysis there is commercial software available, (e.g. GeneSpring), but we have opted for the use of BioConductor, an open source and open development software package (http://www.bioconductor.org/) (meaning it's free). BioConductor is fully integrated into the R environment (http://www.r-project.org/).

Why microarrays. It is clear that microarray technology has great advantages in terms of high-throughput and global analysis. Once a microarray or 'chip' is designed for the complete transcriptome of an organism, arrays may be produced over and over again with little cost, enabling researchers to investigate cellular responses to a wide variety of stimili (conditions).

[1] http://berry.engin.umich.edu/oligoarray2/
[2] http://www.api.com/
[3] http://www.spotfire.com/

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However, especially with large genomes, like is the case for *R. metallidurans* with nearly predicted 7,000 genes, whole-genome arrays represent a major financial investment at a cost of approximately 12 euro per spotted probe. Thus, expression studies may focus on particular sets of genes instead (see below). The technology is highly sophisticated and requires special robotic equipment for spotting and additional equipment for signal scanning and image analysis. Also, the software needed for down-stream analysis can be rather expensive though free software for statistical analysis is also available. In general, a good expertise in statistics and programming with a good knowledge of the various software packages is an absolute prerequisite.

Microarray design: which genes to study? For *R. rubrum* there are no immediate plans for a whole genome chip. Consequently, a selection of genes must be made. This approach is obviously biased and depends on the availability of previous data. Little is known about stress response in *R. rubrum* and literature on this subject is rather limited. Moreover, very few genes involved in stress response, if any, have been identified for this organism. Nevertheless, it is possible, based upon protein sequence similarity searches, to identify candidate genes involved in various stress responses. The procedure entails the identification of stress-related genes in other organisms – i.e based on experimental evidence presented by other research groups – and performing a similarity search by BLAST (McGinnis & Madden, 2004). This has been done by us for *Ralstonia metallidurans*, for which a total of 663 genes were identified as most likely involved in stress-response mechanisms, survival and viability, motility, and metal resistance, by performing BLAST searches against the *R. metallidurans* proteome with particular sets of protein sequences obtained from other species including *Salmonella enterica*, *Ralstonia solanecearum*, *Escherichia coli*, and *Geobacter sulfurreducens*, and selecting homologs in *R. metallidurans* using a cut-off similarity value (selecting 'top hits' only). For *R. rubrum*, a similar approach can be used.

3.4.3 THE EXPERIMENTAL STRATEGY

The production of a microarray or 'DNA chip' for *R. rubrum* would entail:
1) selection of genes for expression analysis:

- the gathering of reliable genome data and placing the entire proteome into one FASTA formatted computer file (e.g. this may be a draft version of JGI)
- selection of genes based on provided annotations and functional class allocation
- selection of additional stress-related genes (or sets of genes of interest) from the genomes of other species and collection of these sequences into one FASTA formatted file.
- selection of genes that serve as control (e.g. housekeeping genes that are not expected to be regulated by the applied stimili, such as *gyrB*, *polB*, etc.)
- performing a multi BLAST similarity search operation between the complete proteome (the *database*) and the selected sets (the *query*)
- choosing top hits, e.g. selecting *R. rubrum* homologs, extracting the corresponding DNA sequences from the complete genome sequence and compile them in one FASTA file.

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2) primer design:
- using the DNA file in FASTA format as input, 50-mer probes are designed with the OligoArray v2.0 software

3) spotting and microarray fabrication:
- a test chip carrying 48 probes will be tested first. If this is sucessful, a larger array will be produced. One glass slide can carry up to 20,000 spots

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Cheung, V., M. Morley, F. Aguilar, A. Massimi, R. Kucherlapati, and G. Childs. (1999). Making and reading microarrays. *Nature Genetics* **21:**15-19.

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McGinnis, S., and T. L. Madden. (2004). BLAST: at the core of a powerful and diverse set of sequence analysis tools. *Nucleic Acids Res* Vol. 32, Web Server issue W20-W25.

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3.5 Genome sequencing of Arthrospira

3.5.1 INTRODUCTION

Planctonic cyanobacteria of the *Arthrospira* genus are presently being sold under the commercial name 'Spirulina' as food supplement. The genome sequence of *Arthrospira* is necessary to (1) verify the nutritional quality on the long term and cultivated under space related environmental conditions; (2) understand the evolutionary mechanisms and characterise the natural biodiversity of the *Arthrospira* genus; (3) define the genomic stability and its potential in space applications as main food source; (4) the comparative genomic analysis to define the biotechnological and pharmacological potentials in light of industrial applications.

3.5.2 THE ARTHROSPIRA SP. PCC8005 GENOME.

The *Arthrospira* strain PCC8005 is easily axenically cultivable. DNA is extractable without too many problems. The GC content of the closely related strain *Arthrospira* PCC7345, is 44.3%, and the genome size is estimated at 5 Mbases.

Planctonic cyanobacteria of the *Arthrospira* genus grow in salty lakes of warm regions. They are mass cultivated in 5 continents and commercialised under the name 'Spirulina' as a food supplement, on the basis of their richness in proteins, vitamins, anti-oxydants, gamma linoleic acids, etc. Likewise they serve for the production of fine chemicals, such as phycobiliproteins. In 2000, the annual production was estimated at 2500 tons with a market of 87 million dollars. Medical studies indicate positive actions of Spirulina against certain virusses, cancers, infections, etc. Spiruline bacteria are recognized by the Food and Drug Administration of the USA as 'Generally Recognized as Safe (GRAS)'. It is for these high nutritional qualities that the *Arthrospira* sp. strain PCC8005 was chosen for photosynthetic producer of oxygen and biomass in the closed loop of the MELiSSA system. While cyanobacteria are generally rich in bioactive components, it is will be necessary to determine the genome of the strains, to prove their nutritional safety and quality during prolonged cultivation under space flight conditions for human and/or animal consumption. It will help revealing the possible presence of genes related to toxicity, characterising the genetic stability and presence/movement of mobile genetic elements (IS, phages, plasmides, ...), discovering new molecules with potential use in the biotechnological/pharmacological industry and study the diversity of the existing *Arthrospira* strains to obtain higher yields of biomass or oxygen. The comparison with a genotypically different *Arthrospira* strain (presently being sequenced in China) will permit to study the structure of the genome and reveal evolutive mechanisms taking place in cyanobacteria belonging to this genus.

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3.5.3 CONSTRUCTION OF A WHOLE GENOME MICROARRAY FOR ARTHROSPIRA SP. PCC8005

Once the complete genome of *A. platensis* is known, its genes are identified, and DNA sequences of genes of interest extracted, 50-mer probes can be designed using the OligoArray v2.0 software as outlined in section 3.2 for. This organism carries about 7,200 genes, thus at least this number of probes are required for a whole genome chip (at a cost of 12 euro per spotted probe). As an alternative, a smaller set of genes my be choosen for expression analysis, as described in section 3.5. Once the probes are purchased, hundreds of slides can be produced.

3.5.4 THE EXPERIMENTAL STRATEGY

It will be necessary to do a minimum of 50,000 runs (with a minimum of 800 bp/run) for the initiale random sequencing. A shotgun cloning will result in fragments with a size of 2 kb, which can be inserted in the sequencing vector. The construction of a banque with larger fragments (6 to 10 kb) is to be inserted in the vector pSXY34. It is forseen that this will provide for a minimum of 5000 readings. Finally the construction of a BAC bank will have to be performed in the vector pBELO, either at the facilities of the MELGEN consortium, or in collaboration with Genoscope. For the closure of the sequence, a collaboration between the Genoscope and the MELGEN team will be aimed at, depending on the number of contigs and the complexity of the gap closure due to repetitions in the genome or low quality sequences. After annotation of the genome, the data can be used for comparative genome analysis, gene expression analysis, presence and movement of mobile genetic elements, etc.

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3.6 Pigment stability/light stress

3.6.1 INTRODUCTION

The photosynthetic compartments of the MELiSSA loop are dependend on light energy to grow. In order to perform photosynthesis, *Arthrospira* and *R. rubrum* posses pigments. Therefore it will be important to investigate the stability of the pigments during prolonged culturing, in reactor conditions and during stress.

3.6.2 PIGMENTS OF R. RUBRUM *ATCC25903*

The photosynthetic pigments of *R. rubrum* are mainly carotenoids belonging to the family of spirilloxanthines; rhodovibrines, which offer the strain their characteristical red-purple culture; as well as bacteriochlorophyl a (Murray et al., 1989). The blue-green color of bacteriochlorophyl a (Bchl a) is masked but could be extracted with methanol. The chromatophores, fragments of the intracytoplasmic membrane, are the seat of the photosynthesis and carry the photosynthetic system (Stanley et al., 1965).

3.6.3 PIGMENTS OF ARTHROSPIRA

Arthrospira contain chlorophyl a and accessory hydrosoluble pigments: red (phycoerythrine) and blue (phycocyanine) phycobiliproteins. Likewise they posses a number of carotenoids (β-carotene, echinone, zeaxanthine, myxoxanthophyle, ...).

3.6.4 DETECTING PIGMENTS, FOLLOW UP OF PIGMENT STABILITY AND LIGHT STRESS USING PROTEOMICS

It will be of prime importance to study the stability of theses complexes that permit the bacteria to grow under light anaerobic conditions. In the bacterial world, photosynthetic pigments are characterized by a complex of proteins, which consist mainly of integral proteins and a small organic part. The stability of these pigment complexes will be investigated and will be monitored at a proteomic level by 2DE analysis for the protein complex part and by MALDI-TOF for the small organic part.

3.6.5 THE EXPERIMENTAL STRATEGY

Study on the photosynthetic protein complex part:
The protein part of the pigment complexes will be analyzed by the 2-DE technology, using the differential extraction method. This differential extraction method has already been tested on *R. rubrum* ATCC25903 in the MELGEN 1 project. The extraction with the buffer 3 containing essentially urea and the detergent ASB-14 allows first to extract the integral protein

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part of the pigment complexes and second to obtain a perfect separation of these proteins by 2-DE. A comparison between different cultures submitted to light stress will be realized.

Study on the photosynthetic small organic complex part:
Our approach will be inspired by the work on green bacterial chlorosomes of Persson *et al.* (2000) who performed the detection of bacteriochlorophyll a and all the major homologs of bacteriochlorophyll c by applying a small volume (1ul) of a concentrated suspension of isolated chlorosomes directly to the target of the MALDI-tof.

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3.7 AFLP optimisation on the ABI sequencer

3.7.1 INTRODUCTION

The high resolution DNA fingerprinting method AFLP has been discussed in detail in TN70.2, section 5.3. and its protocols have been described in TN70.3, section 5.4. In short, AFLP is a method to generate a subset of amplified fragments from genomic DNA without prior knowledge of DNA sequence. First, genomic DNA is digested with a combination of 2 restriction endonucleases. This is followed by the addition of short customised double stranded DNA 'adapters' that have ends that are compatible with the restriction ends of the genome fragments. Thus, all restriction fragments become 'tagged' with adapter ends of known sequences and all fragments can be amplified using PCR primers that are designed to match the adapter ends. During PCR, a selective step is introduced by using PCR primers that extend inward of the tagged fragment with one or more bases. In this way, only a subset of fragments are amplified. During PCR, cy5-labelled nucleotides are built in, so that amplicons can be detected by laser excitation e.g. on an automated sequencing apparatus. Resulting banding patterns are compared to each other by scoring absence and presence of bands (Dice correlation) or by integration of the peak profile (Pearson correlation). Numerical analysis of similarity values between compared patterns are used for dendrogram construction, for instance for phylogenetic analysis or evolutionary studies.

3.7.2 USE OF FAFLP FOR THE GENOTYPING OF MELISSA STRAINS

Originally, the use of the Li-Cor Global IR2 System (www.licor.com) was envisaged, and fluorescent AFLP (fAFLP) was optimized on this apparatus using *R. metallidurans* and *R. rubrum* genomic DNA. Best results were obtained using ApaI-MseI templates (for both organisms) or XhoI-MseI (for *R. metallidurans*) and SalI-MseI templates (for *R. rubrum*) (see Yearly Report 2002, pp. 148). The genomes of both organisms display a GC- content of about 64% but the *R. metallidurans* genome (5.9 Mb) is about twice the size of the *R. rubrum* genome (3.4 Mb). Also, di- and tri-nucleotide frequencies may differ between the two genomes, resulting in less or more DNA cleavage by the used restriction endonucleases and thus leading to different average fragment sizes (size range 50-1000 bp). In general, depending on which primer combination was used for selective PCR, AFLP patterns consisting of 40-50 fragments with average size of ca. 500 bp were obtained. This is ideal for the 5.5% polyacrylamide slab gel format of the Li-Cor Global IR2 System used at the University of Liege, with optimal spread and readout of fluorescent signals.

Although the Li-Cor apparatus has certain advantages, such as the wide fragment size range (i.e. high resolution), the remote control of operation and handling of data, and the provision to actually isolate DNA of interest directly from the gel matrix (e.g. for cloning), there are also some important drawbacks. First, slab gels are ultra-thin (0.2-0.25 mm) making sample loading cumbersome. Second, the used Global IR2 model does not allow internal standards with a second fluorophore, thus pattern analysis relies on the use of external

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standards added to the experiment at regular intervals across the width of the gel. For these reasons, we want to perform AFLP analysis on a more recent apparatus that makes use of capilary electrophoresis and allows simultaneous detection of two fluorescent signals. Such an apparatus is present at the SCK-CEN in the form of an ABI 310 Genetic Analyzer from Applied Biosystems, Foster City, CA (www.appliedbiosystems.com). The use of the ABI 310 equipment will require a slightly different protocol as compared to the Li-Cor. First, ABI technology detects other fluorophores, Cy-3 and Cy-5, which means that new fluorescent primers will need to be tested (i.e. differences in signal strength). Also, the optimal size range for fragment separation on a capilary system is 10-500 bp (as compared to the 50-1000 bp range for the slab-gel based Li-Cor). Consequently, adjusted protocols for the ABI 310 will be applied to assure that evenly distributed but adequately complex banding patterns (actually, peaks in fluorograms) are obtained in a reproducable fashion. Once these protocols are optimized, fAFLP analysis on the ABI 310 will have a much higher and faster throughput as up to 96 samples can be processed in one single run.

3.7.3 THE EXPERIMENTAL STRATEGY

In essence, AFLP could be used for the following:
(1) monitoring of genomic rearrangements and mutations as a consequence of long-term culturing.
(2) investigations on the genetic stability of bacterial strains when exposed to stress, in first instance radiation.
(3) uptake of foreign DNA, i.e. by horizontal gene transfer
Note 1: To enhance the capability of AFLP to detect genomic changes, multiple primer combinations will be deployed.
Note 2: to follow specific events of genome rearrangements, gene specific primers my be used in combination with a AFLP selective primer (for example, a primer that is specific for a transposon gene – movement of the transposon would then be easily registered by changes in the AFLP banding pattern)

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