

Technical Note TN 70.8
Part B: Axeny in long term microbial cultures. Review and trade-off of the technical methods

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ABBREVIATIONS

BHR	Broad-Host Range
BLAST	Basic Local Allignment Search Tool
COGs	Clusters of Orthologous Groups
CV	Coefficient of Variation
dNTPs	Deoxynuleotide triphosphates
EDTA	Ethylene-DiamineTetraAcetic acid
EtBr	Ethidium Bromide (2,7-diamino-10-ethyl-9-phenyl-phenanthridium bromide)
FCM	Flow CytoMetry
HPLC	High-Pressure (or High-Performance) Liquid Chromatography
ICM-MS	Intact cell MALDI_TOF MS
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time-of-Flight
MCP	Micro-Channel Plate detector
MS	Mass spectrometry
NCTC	National Collection of Type Cultures (Colindale, UK)
OD	Optical Density
PCR	Polymerase Chain Reaction
PPM	Parts Per Million
rDNA	rRNA encoding DNA
RDP-II	Ribosomal Database Project (version 2)
RPM	Revelations Per Minute
SAPD	Surface Accessible Proteins Detection
SCM-MS	Supernatant MALDI-TOF MS
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TCA	TriChloroacetic Acid
TEM	Transmission Electron Microscopy
TLF	Time Lag Focusing
Tris	Tris(hydroxymethyl)-aminomethane
UV	Ultra Violet

1. Introduction

The purpose of this Technical Note is to review possible methodologies for axenicity studies on the C2, C3, and C4 compartments of the MELISSA loop.

2. Proteomic analysis

2.1. Outline and principle

Wole-proteome analysis (a proteome being defined as the expressed protein complement of an entire genome), better known as '**proteomics**', holds a key position in modern biology. This discipline recently emerged from the decades-long work on comprehensive protein visualization on two-dimensional gels, which was revitalized by developments in biological mass spectrometry and the growth in searchable sequences databases. Proteomics adds value to these databases by providing tools for the parallel separation and **large-scale identification of proteins** (i.e. high-throughput bacterial membrane protein identification) (**Fig. 2.1.**).

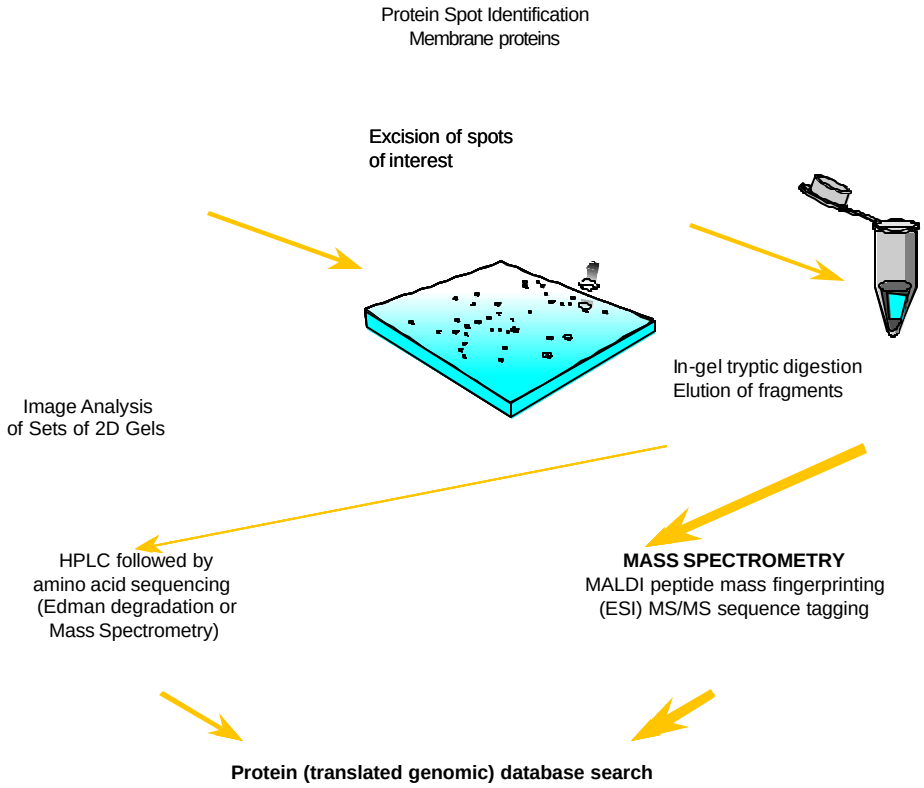

Fig. 2.1.: Strategy for high throughput protein identification

Over the past ten years, mass spectrometry has become the technique of choice for protein or molecules characterization. The reason is the development of new methods for the ionization of proteins and peptides, especially matrix-assisted laser desorption-ionization and electrospray ionization. In the field of proteomics, the technique of MALDI-TOF MS is particularly suited to **high throughput identifications of low femtomole level protein** digest samples (**Fig. 2.2.**).

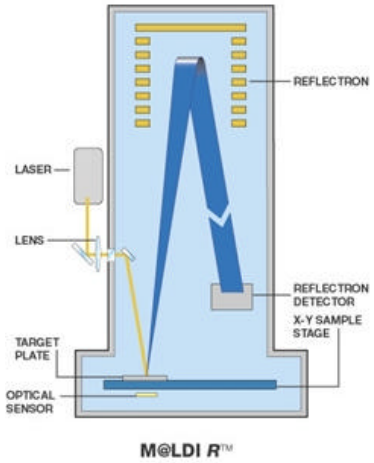

Fig. 2.2. : Reflectron M@LDI Instrument Description

The samples are excised from 2D electrophoresis gels or obtained by high-pressure liquid chromatography. The technique routinely provides high mass measurement accuracy (< 10 PPM RMS) which leads to high specificity for identification of proteins from databases. The new reflectron based instrument has been optimized primarily for the function of peptide mass fingerprinting of large arrays of protein digest samples. More recently, MALDI-TOF is also being used to **characterize microorganisms**.

The reflectron system has an effective path length of 2.3 meters (**Fig. 2.2.**). The target plate is held at ground potential and the flight tube and detector assembly are held at - 20kV. "Time lag focusing" (TLF) is applied to the source to enhance resolution by velocity focusing, this allows the instrument resolution to be almost independent of the laser fluence - an essential element of automated control. The TLF method has been further modified so that the optimum pulse voltage is the same for both high and low mass peptides. The detector system consists of a 2 inch dual MCP (Burle, MA) .

One of the most contentious issues in MALDI (matrix assisted laser desorption ionization) is the role of the matrix in the ionization process. When the laser strikes the matrix crystals, energy deposition is though to cause rapid heating of the sample-crystals brought about by matrix molecules emitting absorbed energy in the form of heat. Laser ionizes sample by 3-ns-wide pulses. Ions are accelerated into the flight tube by accelerating voltage. Since they are allowed to drift through the field-free region to the detector, **they separate according their mass-to-charge ratios**. Lighter ions drift more quickly, heavier more slowly (time of flight -TOF) (**Fig. 2.3.**).

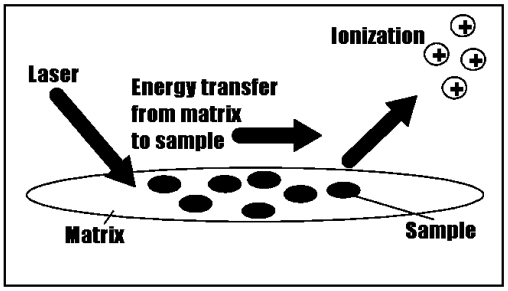

Fig. 2.3.: Schematic representation of the ionization process.

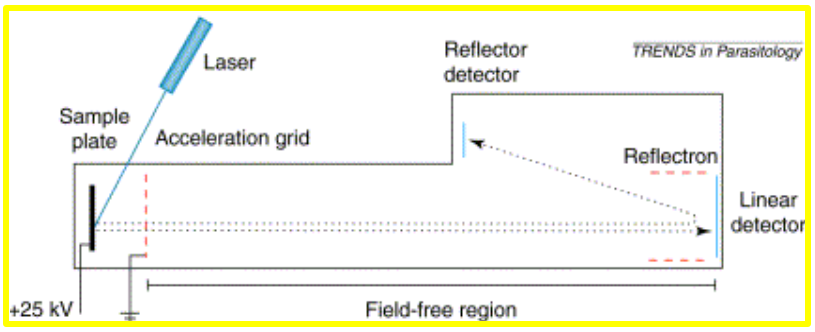

Fig. 2.4.: Schematic representation of MALDI-TOF

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 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 (**Fig. 2.5.**).

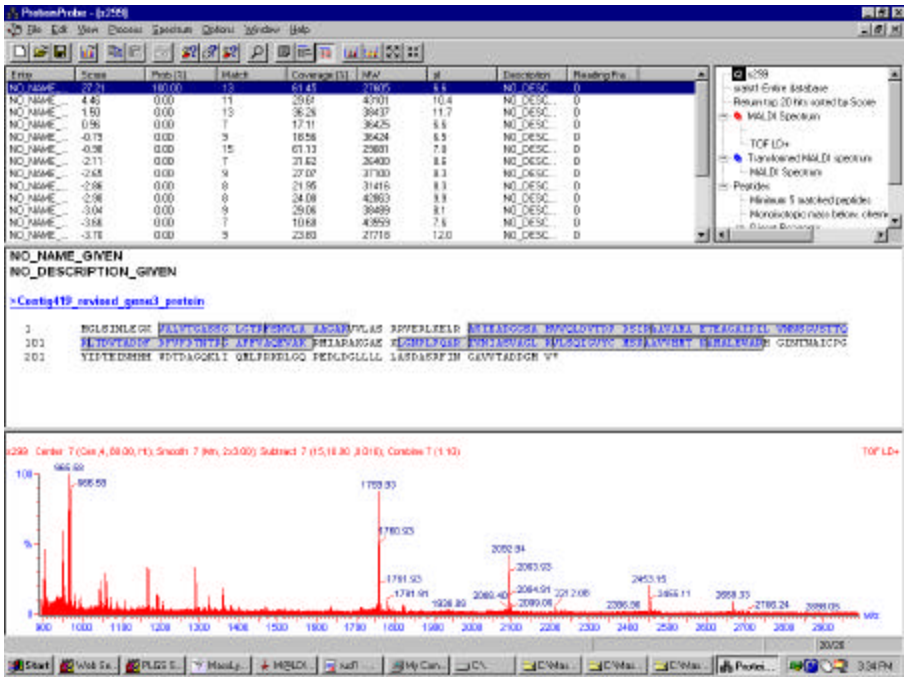

Fig. 2.5.: Example of a mass fingerprint.

This mass fingerprint is characteristic of the particular species, and in some cases differences at the strain level can be observed. Automated collection of mass spectral data from 96 well plates,in less than 30 seconds per sample necessitates the implementation of customised software to analyse and report the data produced in real time. Innovative spectral matching software, which employs a probabilistic search algorithm, is used to match these 'bacterial mass

fingerprints' against the database of quality-controlled reference spectra. The best match, with a probability score, and the genus, species and strain of the organism is reported.

2.2. Detection of contaminations by mass spectrometry

Todate, various proteomics techniques exist (reverse phase capillary HPLC, electrospray ionisation mass spectrometry, gas chromatography-tandem mass spectrometry, MALDI-TOF) that offer new and refined developments to detect microbial contamination. Here we focus on MALDI-TOF.

2.2.1. Intact cell MALDI-TOF-MS (ICM-MS)

Mass spectrometry combined with novel bio-informatic approaches provides a powerful new strategy for the rapid speciation and typing of microorganisms. This revolutionary concept of Bacterial "Mass-Fingerprinting" offers greater sensitivity, selectivity and speed of analysis as compared to classical identification methods that are currently used in clinical microbiology, food science, biotechnology, water quality and pharmaceutical analysis.

Intact cell MALDI-TOF-MS (ICM-MS) applies established biopolymer Mass Spectrometry technology to the analysis of intact bacteria. This method allows the rapid sampling of a population of macromolecules expressed on the surface of bacteria and characterisation of these molecules by molecular weight. The resulting mass spectrum provides a unique physico-chemical fingerprint for the species tested. Mass-Fingerprints of unknown species can be reliably matched against databases of quality controlled reference mass spectra leveraging this simple analytical method into a powerful new tool for real-time detection and sub-typing of bacteria.

Mass-Fingerprinting is very rapid - the entire process from sample preparation to result takes only a few minutes for each test microorganism. Sample preparation is quick and easy - intact cells from primary culture are smeared across a stainless steel target plate and allowed to co-crystallise with a UV-absorbing matrix. After drying, the target is placed into the MALDI-TOF mass spectrometer. The microorganisms in the matrix are illuminated with a pulse from a nitrogen laser (337 nm). The matrix absorbs energy from the laser and macromolecules from the surface of the microorganisms are desorbed and ionised. The resulting ionised macromolecules are mass analysed and the results reported as a mass spectrum, i.e. a plot of mass (X axis) versus abundance (Y axis).

The Mass-Fingerprint of the test microorganism is then submitted to the MicrobeLynx™ search algorithm, which challenges an appropriately selected database from a range of quality controlled bacterial reference mass spectra. A bacterial fingerprinting database (>1500 different bacterial species) is now accessible from the Manchester Metropolitan University in collaboration with the National Collection of Type Cultures (NCTC), Central Public Health, Colindale, UK. This technique could be used to characterize possible contaminations present in the Melissa compartments (Bright *et al.*, 2002). The application of this technique to viruses, bacteria, fungus and spores was reviewed by Fenselau et al. (2001) and Lay (2001). Each bacterial Melissa compartment will be characterized by a specific fingerprint.

2.2.2. Supernatant cell MALDI-TOF-MS (SCM-MS)

Mass spectrometry can be used to isolate, quantify and identify proteins (extracellular proteins : toxins, enzymes…) that may be characteristic of some contaminants. This was applied to the exoproteins of a clinical isolate of *Staphylococcus aureus* where 3 exoproteins and 3 toxins were found (Kawano *et al.*, 2000). To concentrate the bacteria from diluted samples before analysis by MALDI-TOF MS, lectin-derivatized surfaces were used (Bundy & Fenselau, 1999). To study bacteria in dust, 3 markers were analysed by GC-MS by Szponar & Larsson (2001). Ergosterol was a marker of fungal biomass (Saxena *et al.*, 2001), muramic acid indicated peptidoglycan (and thus bacteria), and 3-hydroxy fatty acids was a marker of endotoxin. This method allowed to detect trace levels of these compounds. As in the intact cell mass spectrometry technique, the supernatant, mainly its protein content, is a characteristic of one bacterial species . Until now, the protein content of the different bacterial supernatants has been characterized by two-dimensional gel electrophoresis. Mass spectrometry is also able to characterise the protein supernatant of bacterial cultures by a similar way as the ICM-MS technique. Each bacterial Melissa compartment, especially of the compartment III, will be characterized by a specific supernantant fingerprint.

2.3. Membrane or surface accessible proteins detection (SAPD)

Flow cytometry has great potential as a rapid, automated tool for ecological studies of microorganisms. It is used for detection and characterization of aquatic bacteria, analysis of bacterial populations present in soils and sediments, detection of bacterial food contamination, analyses of intestinal flora and drinkable water. Flow cytometry can detect and separate specific populations of bacteria by using the cell sorting facility. Using an antibody against a specific surface bacterial protein, it will be possible to increase the detection of contaminants in a specific culture, for instance in a particular Melissa compartment. The proteomic approach is one of the best approaches to identify a typical surface protein for each Melissa bacterial culture. In the first step, bacterial surface proteins are labelled by a highly specific surface probe. After extraction, these proteins are then separated by two-dimensional gel electrophoresis and identified by mass spectrometry.

2.4. Microbial sampling requirements

2.4.1. Number and volume of samples required for each time point, frequency of sampling

- **ICM-MS technique:** 3 samplings (1 mL) every three months
- **SCM-MS technique:** 3 samplings (20-30 mL) every three months. The volume of each supernatant culture has to be evaluated primarily,especially for the compartment III.
- **SAPD technique:** 3 samplings (5-10 mL) of the axeny or inoccula cultures (especially for the compartment III and the *Arthrospira* compartment).

2.4.2. Special considerations when taking the sample

- ICM-MS ; SCM-MS and SAPD techniques : ideally, all experiments are to be performed in sterilized polypropylene test tubes. Liquids must be transferred using sterilized graduated plastic pipettes.

2.4.3. Sample preparation just after the sampling

- ICM-MS and SAPD techniques: for the intact cell analysis and the surface protein detection, the samples do not need to be submitted as a specific preparation. A sterile storage of the samples at 4 °C is recommended.

- SCM-MS technique: The samples will be sterilised with specific filtration units (0.2um) (ex: Nalgene disposable filter ware : 150mL filter unit with sterile receiver) just after the sampling. The filtration has to be done in the sterile hood. Before the filtration, a protease cocktail inhibitor (1 tablet for 25 mL of supernatant) is added to the supernatant (in the sterile hood). After filtration, samples are kept at – 20 °C.

2.5. General precautions

The proteome of a bacterial species could be modified by proteases present in the culture or in the supernatant. Wherever possible, a protease cocktail inhibitor (example : complete™ mini EDTA free, Roche) will be used and work will be done at 4 °C. Isolated proteins (ex : filtrated supernatant) can be temporarily stored on ice or refrigerated (2-8 °C) or frozen (-80 to -20 °C) for long-term storage. Storing protein can cause protein precipitation, especially if the protein solution is repeatedly going through freeze-thaw cycles. Thus, it is recommended to freeze multiple aliquots of each preparation.

2.6. General procedures

The protein concentration will be estimated by the classical Bradford technique using BSA as standard. This procedure will be used to determine the required volume to the SCM-MS technique.

2.6.1. Short term and long-term storage procedures

- ICM-MS and SAPD techniques: A short-term storage is available at 4 °C.

- SCM-MS technique: Short and long-term storages are available at -20 °C or -80 °C if the samples are prepared as indicated above.

2.6.2. Target selection/Validation

Which molecules are being targeted?

Flow cytometry can detect and separate specific populations of bacteria by the application of cell sorting. Using an antibody against specific surface bacterial protein would improve the detection of contaminants in a specific culture, for instance in a Melissa compartment. The proteomic approach is one of the best approaches to identify a specific surface protein for each Melissa bacterial culture. In the first step, bacterial surface proteins are labelled by a specific surface probe (Sabarth, 2002). Bacteria were suspended in ice-cold PBS (Phosphate buffer saline), 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ at an optical density at 600nm of 2.5-3.5. The bacteria were surface-labeled by incubation with 200uM (final concentration) sulfosuccinimidyl-6-(biotinamido)-hexanoate (s-NHS-LC-biotin (Pierce)) for 30 min on ice. The reaction was stopped by adding two volumes of TNKCM (50mM

Tris , pH 7.4, 100mM NaCl, 27 mM KCl, 1mM CaCl2, 0.5 mM $MgCl_2$). After 10 min incubation at room temperature, the bacteria were sedimented by centrifugation by centrifugation at 3500 g for 10 min and washed three times with TNKCM. The biotin surface proteins are purified by affinity chromatography on reversibly binding avidin-agarose (Boehringer). After extraction, these proteins are separated by two-dimensional gel electrophoresis and biotinylated proteins were detected on the blots using NeutrAvidin-peroxydase staining (Pierce) and chemiluminescent visualization (ECL, Amersham). Proteins will be identified by mass spectrometry. The specificity of these surface proteins can be further analysed by comparison with the genes included in the different genomic and proteomic databases.

Validation of analytical methods

a) ICM-MS technique:

So far, this technique has been validated to characterise and identify different bacterial species from homogeneous cultures. A new fingerprint database is now accessible (a crossection of bacterial species present in this database is shown in **Table 2.1.**). The mass spectral pattern remained constant for variations in the amount of sample applied to the target plate, variations in sample storage and revival, and for sample storage on target plates. Reproducibility is also observed for repeated experiments on different instruments at different laboratories.

species	nr.	species	nr.	species	nr.	species	nr.
Acinetobacter	3	*Clostridium*	75	*Lactococcus*	1	*Prevotella*	11
Actinobacillus	6	*Corynebacterium*	9	*Legionella*	7	*Porphyromonas*	5
Actinomadura	1	*Edwardsiella*	3	*Listeria*	17	*Proteus*	24
Actinomyces	16	*Eikenella*	1	*Listonella*	2	*Providencia*	13
Aerococcus	1	*Enterobacter*	20	*Mannheimia*	3	*Pseudomonas*	22
Aeromonas	12	*Enterococcus*	30	*Micrococcus*	1	*Psychrobacter*	3
Alcaligenes	1	*Erysipelothrix*	1	*Mistsuokella*	2	*Rothia*	1
Arachnia	1	*Escherichia*	48	*Mobiluncus*	6	*Salmonella*	19
Arcanobacterium	3	*Eubacterium*	2	*Moraxella*	14	*Serratia*	16
Bacillus	17	*Finegoldia*	1	*Morganella*	6	*Shewanella*	1
Bacteroides	29	*Fluoribacter*	3	*Mycobacterium*	8	*Shigella*	2
Bifidobacterium	3	*Fusobacterium*	10	*Neisseria*	36	*Sphingomonas*	1
Bordetella	3	*Garderella*	1	*Nocardia*	2	*Staphylococcus*	40
Brevibacillus	1	*Gordonia*	1	*Oligella*	1	*Streptococcus*	42
Brochothrix	1	*Haemophilus*	18	*Paenibacillus*	3	*Tatumella*	1
Burkholderia	7	*Hafnia*	3	*Pantoea*	1	*Tissierella*	1
Camplyobacter	49	*Helicobacter*	3	*Pasteurella*	16	*Veillonella*	2
Cardiobacterium	1	*Klebseilla*	29	*Pediococcus*	3	*Vibrio*	16
Cedecea	3	*Kocuria*	3	*Peptostreptococc.*	6	*Virgibacillus*	1
Chromobacterium	1	*Kurthia*	2	*Photobacterium*	2	*Walinella*	2
Chryseobacterium	3	*Kytococcus*	1	*Plesiomonas*	2	*Yersinia*	11
Citrobacter	14	*Lactobacillus*	6	*Porphyromonas*	2		

Table 2.1.: crossection of species and strains present in the ICM-MS fingerprint database

The possible detection of contaminations in Melissa compartments by ICM-MS will be validated in a pilot study. In a first step, specific MS fingerprints of *Arthrospira* and *Ralstonia metallidurans* cultures will be characterized. After centrifugation (5000 rpm during 30 min at 4 °C), the pellet will be laid directly with a Maldi-tof matrix on the sample plate. A characterisation of intact bacterial fingerprints will be done in different culture time points. This approach will be compared to the flow cytometry approach on the same samples.

In the second step, the *Arthrospira platensis* and *Ralstonia metallidurans* cultures will be "spiked" i.e. artificially contaminated with other bacteria: for example *Arthrospira* with *Ralstonia* and *Ralstonia* with *Arthrospira*. Different concentrations of the contaminant bacteria will be tested. This study will allow us to assess the sensitivity and the reproducibility of this method.

b) SCM-MS technique :

A similar approach than ICM-MS technique will be used but applied to the culture supernatant. In the first step, a specific protein MS fingerprint of the different bacterial culture supernatants will be characterized. After filtration at 4 °C, the protein in the supernatant will be precipitated by TCA(trifluoroacetic acid) precipitation (10% V/V TCA, overnight at 4C). After centrifugation (15000RPM, 1h, 4C), the pellet will be laid directly with a Maldi-tof matrix on the sample plate. A characterisation of bacterial protein supernatant fingerprints will be done in different culture time points. This approach will be compared to the flow cytometry approach on the same samples.

In the second step, the *Arthrospira* and *Ralstonia metallidurans* cultures will be once again "spiked" , i.e. *Arthrospira* with *Ralstonia* and vice cersa. Different concentrations of the contaminant bacteria will be tested. This study will allow us to assess the sensitivity and the reproducibility of this method.

c) SAPD technique :

This technique has been validated for a large number of bacteria (Sabarth, 2002). Briefly, In the first step, bacterial surface proteins are labelled by a specific surface probe (Sabarth, 2002). After extraction, these proteins are separated by two-dimensional gel electrophoresis and identified by mass spectrometry. In this context, a specific surface membrane two-dimensional gel electrophoresis from the different Melissa strains will be realized. After separation, surface proteins will be characterized by Edman degradation or mass spectrometry techniques.

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3. Amplification of marker genes using the polymerase chain reaction (PCR).

3.1. The principle of PCR

The polymerase chain reaction (PCR) is a technique that mimics nature's way of replicating DNA. Perceived for the first time in 1985 by Kary Mullis and colleagues at Cetus, PCR has been adopted as an essential research tool because it can take a minute sample of genetic material and duplicate enough of it for detailed study. PCR starts with the DNA sample, which is put in a reaction tube along with **i) primers** (short, synthetic pieces of single-stranded DNA that exactly match and flank the stretch of DNA to be amplified), **ii) deoxynucleotide triphosphates** (dNTPs, the building blocks of DNA), **iii) buffers** and **iv)** a heat-resistant enzyme (**DNA polymerase**). Heating the mixture separates the strands of DNA (**Fig. 3.1.**). Then, at varying temperatures, the rest of the components in the mixture spontaneously organize themselves, building a new complementary strand for each original. At the end of each cycle the DNA count has doubled. If you start with one DNA molecule, at the end of 30 cycles (only a few hours later) there will be about a billion copies (assuming excess of reaction components). In practice this means that from very minute amounts of starter material (e.g. 1-2 ng), suffient amounts of amplified product (or 'amplicon') in the range of 1-5 µg can be obtained, for instance allowing its direct visualisation by simple staining and agarose gelelectrophoresis.

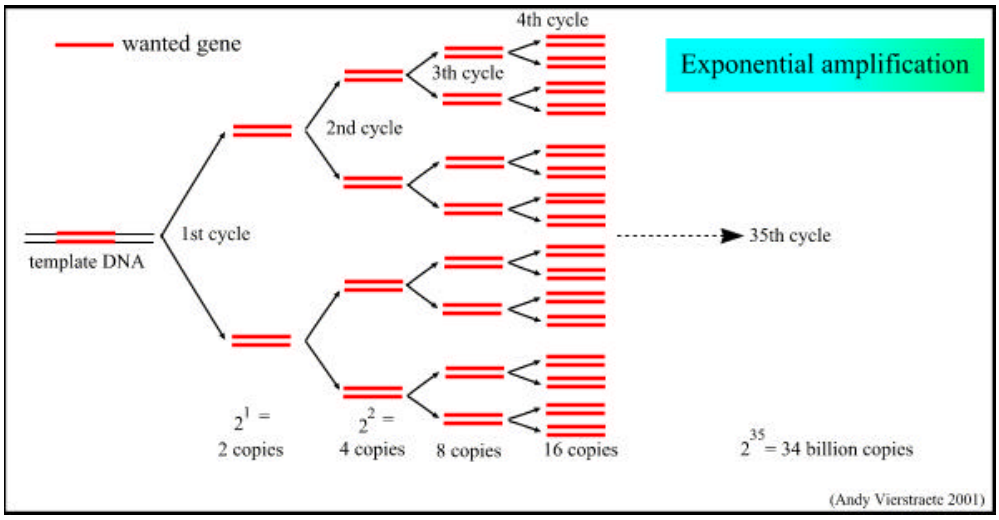

Fig. 3.1.: Principles of the PCR reaction

Today, PCR plays an essential role in DNA sequencing technologies, forensic sciences, genotyping methodologies, and many other facets of molecular biology, medicine, and biotechnology. Over the past decade, several thousands of scientific papers have been written describing new PCR applications, including *in vitro* mutagenesis, gene cloning, and microbial identification.

3.2. Technical aspects

Basically, any of the four reaction components, template, primers, dNTP's, and buffer(s), may vary from reaction to reaction, depending on the application. Although high quality **DNA template** is often preferred, many applications only require crudely prepared DNA. Such is the case in various microbial identification and typing schemes, in which cells are lysed by boiling

and nucleic acids are extracted by phenol extraction. In addition, because the target sequence is rarely larger than 2 kb, high molecular weight DNA is usually not required, and DNA may thus be 'broken' or 'sheared'. Impurities that are co-extracted during the template preparation are often tolerated (within certain limits), as long as they do not severely affect the normal function of the **DNA polymerase**. The presence of enzyme inhibiting compounds will result in lower yields or may become detrimental to the reaction. If inhibition of PCR is suspected, an additional clean-up by extraction (chloroform:iso-amyl alcohol 49:1) followed by ethanol precipitation is recommended. A variety of PCR additives and enhancing agents have been used to increase the yield, specificity and consistency of PCR reactions. Whilst these additives may have beneficial effects on some amplifications it is impossible to predict which agents will be useful in a particular context and therefore they must be empirically tested for each combination of template and primers. Some of the more popular of these additives are dimethyl sulfoxide (DMSO), formamide, bovine serum albumin (BSA), and non-ionic detergents e.g. Triton X-100, Tween 20 or Nonidet P-40 (NP-40). The presence of some salts, especially NaCl and KCl, have a profound effect on DNA reassociation kinetics and primer-to-template hybridisation. In addition, $MgCl_2$ plays an important role in the sensitivity and activity of the DNA polymerase and consequently, special **buffers** have been devised. These buffers contain the necessary monovalent (KCl) and divalent ions ($MgCl_2$, $MnCl_2$) and keep the reaction at a certain pH through the presence of Tris(hydroxymethyl)-aminomethane. Commercially obtained **deoxynucleotide triphosphates** (dNTPs) are usually of very high quality and are not suspect to affect PCR. However, excessive dNTP concentrations can increase the error rate and even inhibit the DNA polymerase (by chelating Mg^{++}). Lastly, physical reaction conditions are very important for a successful PCR. A well choosen **annealing temperature**, at which the primer optimally hybridises to the template, is of paramount importance. This temperature is dependent on a number of factors, including KCl concentration and primer design and can be calculated (using specialised software; see further) or emperically determined. Another variable is the number of cycles which is usually kept at 30, but this number may be increased when extreme low amounts of starting material is being used.

It is clear that PCR conditions often need to be tailored to the requirements of the particular application. The optimal conditions to amplify rRNA-encoding DNA (rDNA) may be determined experimentally by using reference DNA. The currently available equipment used for PCR is generally of high standard and most machines are equipped with a heated lid, as to avoid evaporation of the reaction mix and ensuring a uniform temperature throughout the reaction. Other PCR machines allow extensive testing of reaction parameters, including control of the heating block by temperature gradients or very fast operation with high accuracy using capilary technologies.

3.3. Microbial sampling requirements

It is an absolute prerequisite that growth of experimental and reference cultures and sampling from the reactors for PCR reactions are carried out in a controlled and sterile way, as even minute quantities of contamination, in the order of nanograms of free DNA or as few as 10-100 individual cells, may give false signals in the PCR results. For cultures, one should work in a laminar flow hood, using sterilised (e.g. autoclaved) material. For taking samples from the MELISSA reactors, different strategies need to be deployed, depending on the compartment, but great care must be taken that at no time there is contact with personel, and as little as possible contact with the open environment (e.g., by treating reactor outlets and recipient openings with a blue flame during the sample taking).

After sampling, the cells must be fixed in order to enable the preservation of the nucleic acids (containing the target sequence for PCR). Several methods are possible.

1) The best method is freezing, an easy method when the subsequent steps of analysis are carried out in the same laboratory. The practical drawback of this method when the DNA extraction and PCR steps are performed in a different country (as for the samples of the pilot plant of Barcelona) is that transportation should be done in dry ice, a costly and not very flexible procedure.
2) A practical alternative is the fixation with 70% ethanol. This is also the concentration where nucleic acids are precipitated and the ULg 's lab has good experience with that method for preserving the nucleic acids of cells and sending them by normal post at ambient temperatures.
3) Molecular biology companies have designed reagents for stabilizing RNA, as RNAlater (Ambion Inc., USA), of secret composition, that should be able to preserve the nucleic acids when sampling in places where there is no possibility to freeze (field trips, etc). One can use it for DNA but it depends on the precise method of extraction (e.g. not compatible with the use of phenol).
4) It should be noted that the use of fixatives like formaldehyde should be avoided because they cross-link all cell materials and will inhibit PCR.

3.4. Target selection and validation

Targets of choice in order to generally monitor presence or absence of prokaryotes and eukaryotes are the **ribosomal RNA** (rRNA) encoding genes. Probes can be designed to specifically target narrow to broad phylogenetic groups (from species to domain) by virtue of variable evolutionary conservation within the 16S (prokaryotes), resp. 18S rRNA (eukaryotes) molecules. The 23S rDNA and the 16S-23S intergenic region have also been used for prokaryotic species identification. A number of databases are availaible and can be accessed through the world wide web. The most commonly used are the rRNA WWW Server[1] and the Ribosomal Database Project RDP-II. The latter provides rRNA-related data and tools that are used widely in molecular phylogeny and evolutionary biology, microbial ecology, bacterial identification, microbial population characterization, and in understanding the diversity of life. As a value added database, RDPII offers the research community aligned and annotated rRNA sequence data, analysis services, and phylogenetic inferences derived from these data. These services are available through the RDPII Web site[2]. The 16S and 23S rRNA gene sequences of the principle Melissa strains (*R. rubrum*, *N. europaea*, *N. winogradskyi*, and *A. platensis*) are available, except for *N. europaea*, for which only 16S rRNA sequences are reported – however, the *N. europaea* genome project is reaching completion and information on *N. europaea* 16S rRNA should be released soon or should become available at the genome project's website. The 16S-23S rRNA data can be accessed via the **melgen website** (TN70.1).

Among **other possible target genes** are *gyrB*, encoding the B subunit of DNA gyrase involved in DNA replication, *recA*, involved in DNA recombination and –repair, *glnA*, encoding glutamine synthetase, a pivotal enzyme in nitrogen metabolism, and *dnaK*, involved in the heatshock response (similar to eukaryotic Hsp70). The sequences of these genes already have been used in phylogenetic analyses. **Additional suitable genes** e.g., genes possessing essential attributes such as limited horizontal transmission and presence in all bacterial groups, or in one particular phylum, or only specific for eukarya, may be identified through bioinformatic approaches. However, except for *Nitrosomonas europaea*, very little genomic data are currently available for the principle Melissa strains (TN70.1), which makes the identification of such genes unreliable for the time being. Another option would be to focus on **genes** that are known to be **involved in highly specialised pathways**. This would entail a detailed (literature) study on the

[1] http://www-rrna.uia.ac.be/rrna/index.html
[2] http://rdp.cme.msu.edu/html/

unique physiological characteristics of the Melissa strains, and bioinformatic analyses on the underlying genes. In this context, genes representing important functions in the nitrogen cycle (nitrite reductase, ammonia monooxygenase, nitrogenase, urease) and the carbon cycle (RuBisCO, carbonic anhydrase) could be scrutinised. In the search of a good target sequence however, one should be aware of the possible caveats. For instance, although one would expect to find the ammonia monooxygenase *amoA* gene only in nitrifying bacteria (e.g. specific to C3), no less than 463 AmoA protein sequences are listed in the SYSTERS[3] database of protein clusters, of which 144 belong to the ammonia monooxygenase (EC 1.13.12.-) group, while 305 sequences are found in the anthranilate synthase (EC 4.1.3.27) group. According to SYSTERS, the two groups belong to the same family and are obviously linked by a common functional domain (e.g., they form one family based on BLAST analysis). The former group is only found in nitrifying bacteria (*Nitrosomonas*, *Nitrosococcus*, *Nitrospira*, *Nitrosolobus*, etc.) while the latter group contains proteins from a variety of very diverse genera, including *Mycobacterium*, *Streptomyces*, *Aeromonas*, *Escherichia* , *Halobacterium*, *Synechocystis*, and soforth. Thus, in the case of the *amoA* gene, it is paramount to find the correct part of the sequence in order to specifically design a probe for nitrifying bacteria only [Note: it may even be possible to take this one step further and find a coding sequence (within a given gene) that is highly specific for a particular genus, e.g. by finding DNA sequences that only occur in *Nitrosomonas*] (see also further, **Fig. 3.2.**). In practice this means that, once a suitable target gene is choosen, the protein sequence needs to be checked for multiple domains to ensure that the DNA sequence determined for primer design is derived from the correct domain. A number of **interactive websites** are available to assist in this search:

COGs	http://www.ncbi.nlm.nih.gov/COG/
InterPro	http://www.ebi.ac.uk/interpro/
Pfam	http://www.sanger.ac.uk/Software/Pfam/
Prodom	http://prodes.toulouse.inra.fr/prodom/doc/prodom.html
SMART	http://smart.embl-heidelberg.de/
BLOCKs	http://blocks.fhcrc.org/

Some of the services listed above have links to each other, for instance a Pfam domain notation may be used for a search action in SYSTERS, while other websites offer additional services, such as the BLOCKs CodeHop facility[4] to design a (degenerate) primer from protein multiple sequence alignments.

In conclusion, there are **two major lines** in which marker genes may be used. First, to check the presence (or rather, absence) of certain lineages of prokaryotic and/or eukaryotic organisms using 16S and 18S rRNA probes. This is particular suited to follow possible contamination coming from C1 or from the environment. Second, highly specific probes may be designed based on biosynthetic, catabolic, or regulatory genes unique to one of the Melissa strains, allowing to monitor possible contamination between C2, C3, and C4.

3.5. Primer design

The major steps in probe design are identifying short regions (usually 15–25 nucleotides in length) in a sequence alignment unique to the target group of interest, centralizing mismatches to

[3] http://systers.molgen.mpg.de/
[4] http://blocks.fhcrc.org/blocks/codehop.html

nontarget organisms (where possible), and modifying the sequence to meet probe design criteria such as a minimum melting temperature. There are various **software packages** available that may assist in target selection.

One is **ARB**[5], a UNIX-based, graphically interfaced program for handling large sequence databases (e.g. rRNA data). ARB was developed by Wolfgang Ludwig and colleagues at the Technical University of Munich. Sequences for analysis may be directly downloaded from the internet and data may be imported in many formats, including FASTA and GENBANK. A special feature of ARB is that the alignment is not only sequence based but also takes into account the secondary structure information. This often helps to align gene segments in spite of the fact that these segments may have one or more insertions/deletions (known as 'indels'), simply by knowing which positions contain bases that are paired, and thus should be complementary. The function 'Probe Design' is also a unique feature of this software. It is possible to indicate on a phylogenetic tree, connected to the sequence data, which lineage or organism should be targeted by the probe. It is also possible to allow for mismatches. The program shows the different candidates for probes and one can choose among them on the basis of other criteria (see below). These features makes this program really unique. Another program, **BioEdit**[6] (Fig. 3.2.), has recently been developed by Tom Hall (North Carolina State University). This program primarily functions as a biological sequence alignment editor written for Windows 95/98/NT and has not the same special feautures as ARB. Nonetheless is has several attractive features such as automated BLAST searches (local and WWW), restriction mapping, RNA comparative analysis tools, and translation-based nucleic acid alignment. Both software packages are freely available and can be downloaded from their respective websites.

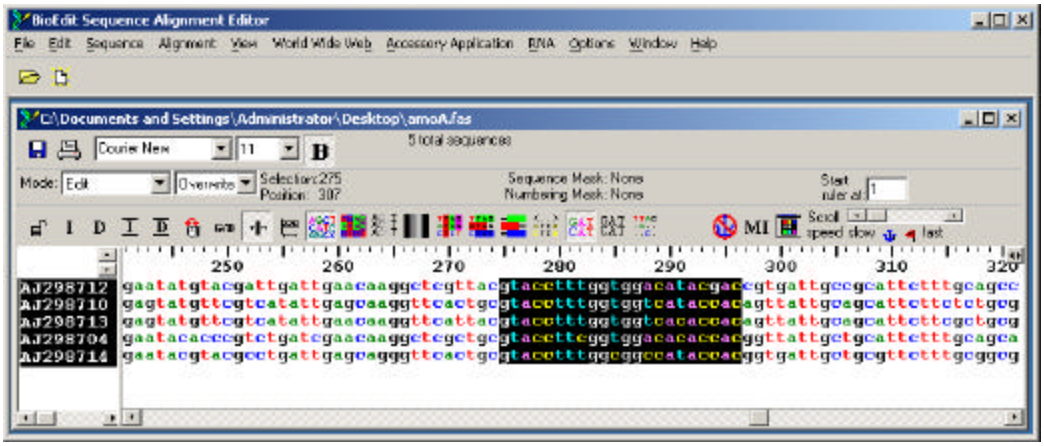

Fig. 3.2.: Alignment of *amoA* gene sequences from five *Nitrosomonas* species (AJ298712, *N. cryotolerans*; AJ298710, *N. europaea*; AJ298713, *N. eutropha*; AJ298704, *N. halophila*; AJ298714, *N. nitrosa*) and putative primer binding site.

On the RDP-II web service (see section 3.4.), it is possible to test the specificity of the choosen probe. This is done on-line using 'Probe-Check' and is very useful to verify ARB produced results. In addition, sequences that were not present in the latest version of the ARB database may still be found on RDP-II, which is updated with a higher frequency.

Once a suitable DNA sequence can be proposed for primer production, several **criteria for proper primer design** need to be checked. The most important issues are length, sequence

[5] http://www.arb-home.de/
[6] http://www.mbio.ncsu.edu/BioEdit/bioedit.html

composition, and experimental conditions. A PCR primer is usually between 15 and 30 nucleotides (nt) long, but the actual length is not important. The **length** of a primer is often adjusted with one or two nt as to compensate for the fact that the two other criteria may not be entirely matched. Sequence **composition** is very important and actually follows common sense: i) primers with moderate base content are clearly preferred (40-60% GC), ii) stretches of nucleotides (e.g. AAAA) should be kept to a minimum, and iii) the sequence should not contain palindromes (to avoid formation of hairpin loops and primer dimers). In addition, introduction of 'wobbles'[7] for the construction of degenerate primers should not be in excess since degeneracy lowers the annealing temperature and hence specificity. The degeneracy of the primers can be kept "down" by substituting four-base wobbles with inosines. (Example: GGI instead of GGN). An on-line resource is provided by the Weizmann Institute, Israel, to deal with degenerate primers (**CodeHop**, see section 3.4.). For cloning purposes it may also be advisable to use 5'-G residues whenever possible to exploit terminal extendase activity of some DNA polymerases (e.g., for A/T cloning). There are several software packages available that allow proper primer design (e.g. Primer Premier 5 by Biosoft International), but one of the best programs currently available for free is the **Primer3 www-interface**[8] at the Whitehead Institute, MIT. Very recently, a new website, **probeBase**[9], was established by Prof. Michael Wagner and collaborators of the Microbial Ecology group at the TU Munchen. This database contains published rRNA-targeted oligonucleotide probe sequences, DNA microarray layouts, and associated information. An interesting feature of proBase is the possibility to search for target organisms or a particular probe name. In addition, a "search target site" function can assist in the development of new

UNIV1390	Tested for in situ hybridization. ▶ *Glossary
Name: (Alm et al., 1996)*	S-*-Univ-1390-a-A-18
Specificity:	all Organisms ▶ Check specificity using the ARB difference alignment* ▶ Check specificity using Probe Match at RDP II (Michigan State University)
Target molecule:	16S rRNA
Position:*	1390 - 1407
Sequence:*	5'-GACGGGCGGTGTGTACAA-3'
Length:	18
G+C content:	61,1
Tm:*	53
MW:	5598
Formamide (FISH)*:	0%
Reference:	Zheng D., Alm E. W., Stahl D. A. , and Raskin L. (1996). Characterization of universal small-subunit rRNA hybridization probes for quantitative molecular microbial ecology studies. Appl Environ Microbiol. 62: 4504-4513. ▶ Abstract

Fig. 3.3.: Query result from proBase, an on-line resource for rRNA targeted oligonucleotide probes

[7] Degenerate PCR is in most respects identical to ordinary PCR, but with one major difference. Instead of using specific PCR primers with a given sequence, mixed PCR primers are used. That is, if the sequence of the gene to be amplified is unknown, "wobbles" are inserted in the PCR primers where there is more than one possibility.
[8] http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi
[9] http://www.microbial-ecology.de/probebase/

rRNA-targeted oligonucleotide probes while the "find probe set" tool can be used to rapidly retrieve all published probes targeting the user's query sequences (e.g. from a rRNA gene clone library) without prior phylogenetic analysis. The query result also contains usefull information on primer characteristics (**Fig. 3.3.**). proBase currently comprises 533 probes (as of June 4, 2002).

A possible improvement in the overall procedure of PCR may be obtained by using a **nested PCR**. In this approach two pairs of PCR primers are used for a single locus. This enhances specificity as the chance for repeated spurious primer annealing in the same locus is very small. Other improved procedures are the **Hot-start** PCR - to reduce non-specific amplification- and **Touch-down** PCR - to reduce non-specific PCR product.

3.6. Analysis procedures and methods

3.6.1. General precautions

Because the polymerase chain reaction is capable of amplifying as little as a single molecule of DNA, precautions have to be taken to guard against possible contamination of the reaction mixture with trace amounts of DNAs that could serve as templates. Therefor, DNA for PCR is best isolated and purified in a different area as to the PCR sample preparation. PCR sample preparation is best performed in a laminar flow or special room, using a separate set of automatic pipettors and filter tips to overcome aerosol driven contamination. General size 0.2 ml thin-wall eppendorf tubes are always sterilized before use. Negative controls (i.e. reaction mix without DNA) should be run with the cycled samples. The water used in PCR is usually heat-sterilized, distilled water (Millipore) and may have been subjected to an additional UV sterilization step (not yet installed). It has to be emphasized that all three major steps of PCR (DNA preparation, sample preparation and PCR) are best performed in different rooms. In addition, the reagents are aliquoted when received so that each user has his/her own lot of reagents that are easier to control.

Ideally, all experiments are to be performed in lockable sterilized polypropylene test tubes (capacity of 0.2 to 50 ml). Liquids must be transferred by automatic pipettors (Gilson or similar) using sterilized pipette-tips. For volumes larger than 1 ml, sterilized graduated plastic pipettes must be used. All tubes must be sterilized by steam autoclaving (45min, 121 °C).

3.6.2. Some basic procedures

Storage of the isolated DNA:

DNA can be temporarily stored on ice or refrigerated (2 ~8°C) or frozen (-80 ~ -20°C) for long-term storage. Storing genomic DNA at –20ºC can cause shearing of DNA, especially if the DNA is repeatedly going through freeze-thaw cycles. It is therefor recommended to freeze multiple aliquots of each preparation. Storage in 70% ethanol is an option, but this requires a precipitation step prior to the experiment.

Procedure for OD quantification of isolated DNA:

Nucleic acids absorb UV light of 250 to 270nm wavelength, with a maximum at 260nm. The reading at 260nm allows calculation of the concentration of nucleic acid in the sample. An OD of 1 corresponds to approximately 50 ng/µl for double-stranded DNA

and 40 ng/µl for single-stranded DNA and RNA. The ratio between the readings at 260nm and 280nm (A ratio) provides an estimate for the purity of the nucleic acid. Pure preparations of DNA and RNA have ratios of 1.8 to 1.95, respectively. DNA producing a ratio below 1.6 should be re-purified.

Qualitative inspection:

DNA quality can be easily checked on a 0.8-1.0 % (w/v) agarose gel matrix (see further): the primary feature should be a single, high molecular weight band, with as little as possible 'smear'. Prominent smear indicates extensive DNA degradation.

3.6.3. Sensitivity of a PCR analysis

The overall sensitivity of a DNA-based PCR analysis for the detection of microorganism in the environment is determined primarily by the following key elements:

- The nature and size of the analytical sample
- The procedures used for *DNA extraction* from the sample
- The specifics of the subsequent *PCR analysis* performed on the extracted DNA.

Sample

While not technically part of the PCR analysis, the sample can set the actual limit on the sensitivity of each analysis. Any claim related to sensitivity of a specific PCR analysis must take into consideration the homogeneity of the sample matrix, the coarseness of the sample, and the amount of the actual sample (see 3.3.).

DNA Extraction

Extraction protocols must simultaneously address the variables of DNA concentration and the presence of inhibitory compounds for each sample type. In doing so, the protocols must be evaluated in light of two criteria:

- **DNA yield:** The condition and amount of the DNA in submitted samples, as mentioned above, varies widely. Various extraction procedures may be tried i.e. an extraction protocol may be optimized to effectively deliver DNA of adequate purity, homogeneity, and molecular weigth. An inefficient DNA extraction, especially at lower concentrations, will limit the sensitivity of that analysis.

- **DNA purity:** during extraction of DNA various compounds that can interfere with the subsequent PCR detection may be extracted along with the DNA. Lowered efficiency of the PCR reaction may result in reduced sensitivity or even false negative results. Therefore additional purification steps may become necessary, according to the degree of impurities of the initial DNA extract.

PCR Analysis

Once an extraction protocol has been shown to deliver a consistent yield of pure DNA, the next step is to set up a PCR routine to amplify the DNA sequence of interest.

- **Primer sets:** Computer-aided primer design and database research are helpful tools during development of primer systems that are highly specific for their intended target sequence of DNA (see 2.5.). However, before being used, each primer set must be thoroughly validated. This is a process of testing against various reference DNAs (*in vitro* testing, for instance in the range of 0.001 – 20 ng) or on mixed ('spiked') samples containing different quantities of reference cells (*in vivo* testing). Only this validation process ensures that a PCR system does not accidentally produce copies of non-target DNA resulting in misleading artifacts.

- **Number of cycles:** The PCR process works exponentially, so even slight changes in the number of cycles used can have a significant impact on the ability to detect low amounts of target DNA. In a perfect, theoretical world, a PCR of 35 to 40 cycles would be sufficient to amplify single molecules of target DNA to detectable levels.

3.6.4. Visualisation of the amplified products

The standard method used to separate, identify, and purify DNA fragments is electrophoresis through agarose gels. The technique is simple, rapid to perform, and capable of resolving mixtures of DNA fragments that cannot be separated adequately by other sizing procedures. Furthermore, the location of DNA within the gel can be determined directly: Bands of DNA in the gel are stained with the intercalating dye ethidium bromide ; as little as 1 ng of DNA can be detected by direct examination of the gel in ultraviolet light.

Commercially available agarose is not completely pure, it is contaminated with other polysaccharides, salts, and proteins. These differences can affect both the migration of the DNA and the ability of the DNA recovered from the gel (e.g. to serve as a substrate to further enzymatic reactions). When an electric field is applied across the gel, the DNA, which is negatively charged at neutral pH, migrates toward the anode. The electrophoretic migration rate of amplicons through agarose gels is dependent upon three parameters:

The molecular size of the amplicon. Molecules of linear, duplex DNA, which are believed to migrate in an end-on position travel through gel matrices at rates that are inversely proportional to the logarithm of their molecular weights.

The agarose concentration. A DNA fragment of given size migrates at different rates through gels containing different concentrations of agarose. There is a linear relationship between the logarithm of the electrophoretic mobility of the DNA and the actual gel concentration. Thus, by using gels of different concentrations, it is possible to resolve a wide-range of DNA fragments.

The applied current. At low voltages, the rate of migration of linear DNA fragments is proportional to the voltage applied. However, as the electric field strength is raised, the mobility of high-molecular-weight fragments of DNA is increased differentially. Thus, the effective range of separation of agarose gels decreases as the voltage is increased. Gels should be run at no more than 5 V/cm.

The agarose concentration is varied for different fragment ranges. For analyzing 0.1-1 kb sized amplicons usually a 0.8-1% agarose gel is made by dissolving agarose in Tris-Acetate-EDTA (TAE) buffer and heating in a microwave oven. After cooling to about 60°C, ethidium bromide (2,7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide) is added to a final concentration of 0.5 µg/ml. This substance intercalates between stacked bases of DNA. Dye that is bound to the DNA displays an increased fluorescent yield compared to dye that is in free solution. UV-irradiation absorbed by the DNA at 260 nm and transmitted to the dye, or irradiation absorbed at 300 nm and 360 nm by the bound dye itself, is emitted at 590 nm in the red-orange region of the visible spectrum.

A gel loading buffer is applied to 10 µ samples electrophoresis which are run by 70-100 V/20-80 mA for about an hour or at 20 to 30 V overnight. The size of fragments can be determined by calibrating the gel, using known standards (e.g., DNA EcoRI / HindIII digest, Boehringer Mannheim, or 100bp ladder, BioVentures, Inc.), and comparing the distance the unknown fragment has migrated.

3.7. References

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4. Monitoring of plasmid transfer

4.1. Aims

The transfer of genes in the MELISSA loop has to focus

1°) on the capacity of the bacteria of the **MELISSA** loop to transfer genes OR to capture genes OR to act as recipients of foreign genes

2°) on the potential of plasmid-mediated gene dissemination in the MELISSA-loop outside the MELISSA-bacteria

In this part of the WP, we will mainly focus on plasmid-mediated conjugation as the most probable and measurable means of (undesired) gene dissemination but transformation and phage-mediated will be also addressed to some extent.

4.2. The capacity of the MELISSA bacteria to participate in plasmid-mediated gene transfer

4.2.1. Monitoring the presence of a plasmid in a MELISSA strain.

For compartment C2: a plasmid of around 55 kb is expected to be present in most strains of *Rhodospirillum rubrum*, if not all (see TN70.7). The presence of this plasmid has to be checked via a DNA extraction method (alkaline lysis) that puts in evidence the presence of circular DNA on agarose gel electrophoresis. As the first draft of the genomic sequence of *R.rubrum* is now available (4.5 MB in 27 contigs) (since April 15 on the JGI website) , it would be of interest to check the presence of the plasmid or of plasmid-bourne sequences in one or more of these contigs and to check (via PCR) the correlation between any plasmid isolated from alkaline lysis and the genomic sequence.

For compartment C3: there is very little evidence for plasmids in nitrifying bacteria. Yet it has to be checked in nitrifying bacteria using a variety of plasmid extraction methods. It may even be appropriated to validate these extraction methods by introducing a known plasmid in *Nitrosomonas* or in *Nitrobacter*.

For compartment C4: as in C3, although there is abundant evidence for plasmids in Cyanobacteria.

4.2.2. Conjugative properties of plasmids from MELISSA bacteria.

The focus is clearly on the *R. rubrum* plasmid. It should be tested if this plasmid is able to conjugate to a plasmid-free *R. rubrum* (if available) or to other taxonomically distant strains. To check this last point, it will probably necessary to tag the plasmid with a resistance marker. Tagged plasmids from R;rubrum may exist in some laboratory collection. Outside the self-transfer, two important conjugative features have to be tested :

- the mobilisation of a non-transferable plasmid by the *R. rubrum* plasmid in a plasmid-free recipient
- the *R. rubrum* plasmid-mediated retromobilisation of a non-transferable plasmid from any strain into the *R. rubrum* strain itself (plasmid capture , retrotransfer) (For a review on retrotransfer and its ecological significance, see Szpirer *et al.*, 1999) [C.SZPIRER, E.TOP, M.COUTURIER, M.MERGEAY (1999). Retrotransfer or gene capture: a feature of conjugative plasmids, with ecological and evolutionary significance. *Microbiology* 145: 3321-3329]

Special attention has to be given to these features due to their relevance in gene dissemination, especially if C2 comes into contact with external contamination or with C1 microflora.

Testing mobilisation and retromobilisation mediated by *R.rubrum* plasmid will require the use of various mating partners (plasmid-free *R.rubrum*, another more or less closely related strain in α-Proteobacteria (*Rhodobacter sphaeroides*?), *R.metallidurans*, *P.putida* or *E.coli*) and of non-transferable mobilisable vectors provided with appropriate markers.

Broad-host range plasmids from the IncQ family are particularly suitable for this purpose. If a plasmid is to be found in an other MELISSA strain , the same conjugative features of self-transfer, mobilisation and retromobilisation have to be tested as well although it will obviously be quite difficult from the technical point of view

4.3. Plasmid-mediated gene dissemination in the MELISSA-loop outside the MELISSA-bacteria.

4.3.1. Sources of exogenous plasmids.

Two main potential sources should be considered : the C1 compartment and the supernatants of the MELISSA loop if exposed to external contamination or to any rupture of axenicity

4.3.2. Acquisition of plasmids from these potential sources.

This should be tested by mixing the potential sources of plasmids (non-axenic or contaminated MELISSA-loop, non-filtrated supernatant from C1 compartment) with various recipients : the MELISSA bacteria (mainly *R.rubrum* and possibly *Arthrospira* but also bacteria that are known as good recipients of plasmids or that are able to efficiently express foreign genetic bacteria: various strains of *R.metallidurans, P.putida , E.coli* are perfectly suitable for this purpose.

Two categories of plasmids are of special interest in these experiments:

- plasmids carrying selectable markers (resistance to antibiotics, ionising radiation, or heavy metals, the capacity to degrade recalcitrant organics, etc.). In view of biosafety measures for personel in space, the early detection of antibiotics

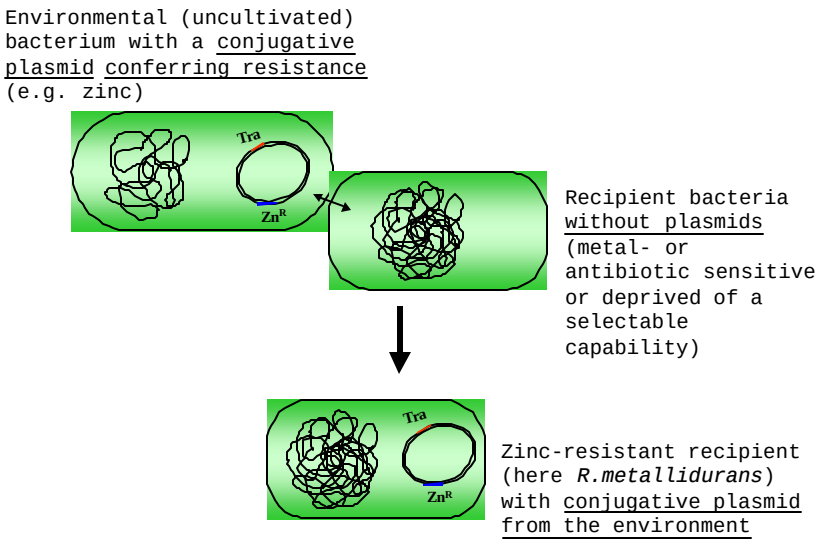

Fig. 4.1.: Direct (biparental) exogenous isolation of environmental plasmids

resistance is of special relevance as new capacities or elevated levels of bacterial resistance against antibiotics may be considered as totally undesirable. In addition, the presence of any other plasmid-borne marker needs to be scrutinised. The acquisition of exogenous plasmids from MELISSA non-axenic or C1 supernatants could be tested by **direct (biparental) exogenous isolation of environmental plasmids (Fig. 4.1)** using *Ralstonia metallidurans*, an organism that displays

resistance to heavy metals, but any other suitable recipient or marker can be tested as well.

- Broad-host range (BHR) mobilising plasmids are selected not because they may carry a resistance or metabolic marker but exclusively for their capacity to mobilise another plasmid (or any mobile genetic element) from one bacterium to an other. Many well-known BHR plasmids display such a property which is of utmost relevance for gene dissemination in confined environments such as the Melissa loop. The presence of BHR mobilising plasmids from Melissa non-axenic supernatants or from the C1 compartment can be monitored by means of triparental matings (Fig. 4.3.). It should be noted that such plasmids with mobilising activities may have no other phenotype or marker and could therefor be considered as being "cryptic" plasmids.

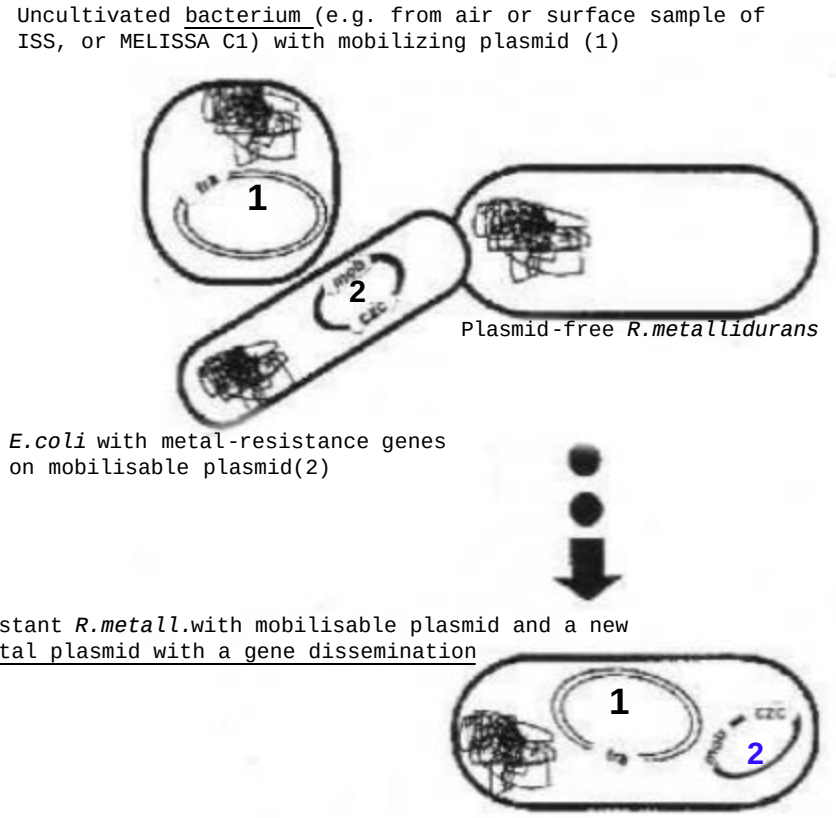

Fig. 4.2.: Triparental matings: an assay for gene dissemination

4.3.3. Subsequent analysis of plasmids.

Any plasmid found in biparental or triparental matings should be thoroughly studied , with special emphasis on the plasmids coming from C1 compartment . The study would include the estimation of plasmid size, its host range especially towards Melissa bacteria

and the presence of some relevant genetic determinants via PCR mediated amplification (virulence markers, resistance to antibiotics, etc….).

5. Analysis by flow cytometry

5.1. A short introduction to flow cytometry.

In flow cytometry, the cells may be alive or fixed at the time of measurement, but must normally be in monodispersed (single cell) suspension. They are passed single-file through a laser beam by continuous flow of a fine stream of the suspension. Each cell scatters some of the laser light, and also emits fluorescent lights excited by the laser which are collected by photomultiplier tubes positioned at different angles relative to the incident light and 90° relative to the jet of the cells (figure 1).

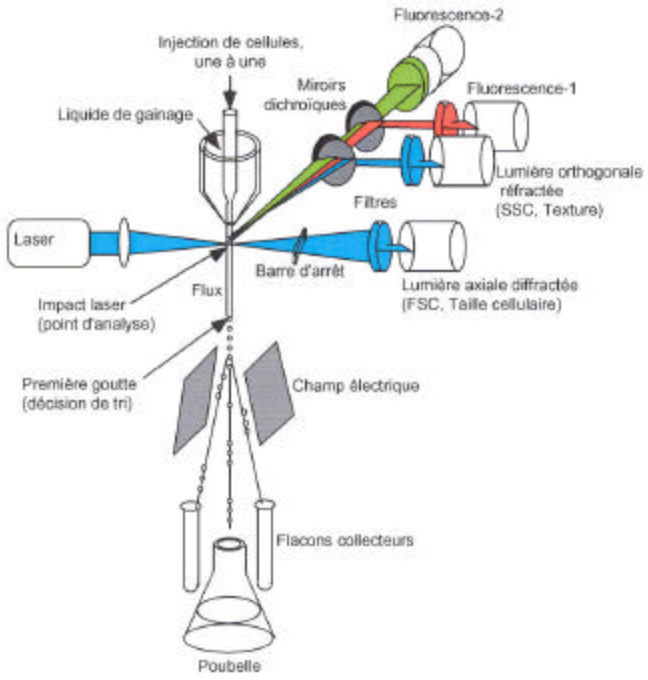

Fig. 5.1.: Individual cells confined within a rapidly flowing jet of water pass a measuring window, at which several parameters can be simultaneously measured (Fig 5.2)

The flow cytometer typically measures several parameters simultaneously for each cell (Fig. 5.1 and Fig. 5.2):

- a low-angle forward scatter intensity, approximately proportional to cell diameter

- an orthogonal (90 degree) scatter intensity, approximately proportional to the quantity of granular structures within the cell
- fluorescence intensities at several wavelengths

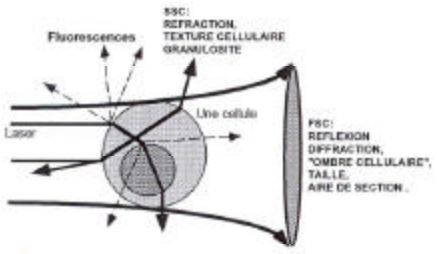

Fig. 5.2.: measurement of various parameters in flow cytometry

Light scatter alone is often quite useful. It is commonly used to exclude dead cells, cell aggregates, and cell debris from the fluorescence data. Fluorescence intensities are typically measured at several different wavelengths simultaneously for each cell. Different cell components are stained with fluorescent dyes and, as the cells pass the window, the dyes are excited with a high-intensity light source. The emitted fluorescence from the dye is proportional to the cellular content of the stained substance. In this way, DNA content (measured as fluorescence from stained DNA for example) can be measured at rates of more than 1,000 cells per second. The method yields unique information about the distribution of a parameter between the individual cells in a population, which provides a different and more informative result than methods in which average values are measured. Fluorescent antibodies are often used to report the densities of specific surface receptors, and thus are useful to distinguish subpopulations of differentiated cell types. By making them fluorescent, the binding of viruses or hormones to surface receptors can be measured. Intracellular components can also be reported by fluorescent probes, including total DNA/cell (allowing cell cycle analysis), newly synthesized DNA, specific nucleotide sequences in DNA or mRNA, filamentous actin, and any structure for which an antibody is available. Flow cytometry can also monitor rapid changes in intracellular free calcium, membrane potential, pH, or free fatty acids.

Flow cytometry involves sophisticated fluidics, laser optics, electronic detectors, analog to digital converters, and computers. The computer records data for thousands of cells per sample, and displays the data graphically.

Analysis Equipment at SCK-CEN: the EPICS XL (Beckman Coulter).

The facility at the laboratory of radiobiology at SCK-CEN provides a Beckman Coulter EPICS XL for analysis of cell samples. This instrument cannot separate i.e. sort cells into different containers based on their properties; the samples are thus consumed and discarded during analysis. The EPICS XL is a closed fluidic system, allowing the analysis of biohazardous samples (such as human blood samples, bacteria) although appropriate precautions should be taken.

The EPICS XL uses an air-cooled argon gas laser, 15 mW output, with a fixed wavelength emission of 488 nm. It has four fluorescence detection channels which simultaneously detect green, yellow-orange, and red light. Fluorescein is used extensively for the green channel, and

phycoerythrin or propidium iodide (a DNA stain) for the yellow-orange channel. Dyes are also available which can be excited at 488 nm yet emit in the red.

The EPICS XL can analyze cell suspensions at the rate of several hundred cells per second. Typically, investigators acquire 5,000 to 15,000 cells per sample. Data are saved to the hard disk of a dedicated computer, where they can later be analyzed with graphics software.

5. 2. Optimised detection of bacterial changes induced by stress, contamination or viral infection.

5.2.1. Optimisation of the flow rate of sample cell inside the flow cytometer

The quality of the results depends on the cell flow rate expressed as cells per minute that passes through the flow chamber inside the flow cytometer. When the rate is too high, the sharpness of the peaks decreases. When the rate is too low, the experimental time is too long. An optimal cell flow rate needs to be determined emperically.

5.2 .2. Discrimination of the MELISSA strains from the background

To decrease to the minimum the level of background noise, the media that are used in order to analyse bacterial populations will have to be carefully filtered (0.22 µm, 1, 2 or 3x). Then, the discriminator has to be set on size (if cells are not stained), on DNA fluorescence (if the DNA of the cells is stained) in order not to visualise background.

5.2.3. Basic detection of differences in size and granularity of the MELISSA strains

Some of the most important work in marine microbiology using FCM includes the ability to detect, count, sort, and identify free living bacteria to help better understand their role in the marine ecosystem. FCM has shown that it is has the ability to identify pico-phytoplanktonic cells. These particular organisms present a great challenge owing to their very small size of 0.2 to 2 µm, and even basic questions about these organisms remain unanswered. However, with natural seawater samples, flow cytometry enables the discrimination of pico-organisms from larger phytoplanktonic cells, photosynthestic prokaryotes, bacteria, and nanoheterotrophs. The interest in these organisms finds its origin in the fact that they act as primary producers and as a food source. Using rRNA oligonuclotide probes, the organisms can be subjected to *in situ* hybridization and based on their different degrees of side scatter the organisms are readily detected. The FCM histograms can be directly examined to aid in bacterial determination.

There is a relationship between the forward scatter (size) and cell mass. In addition, the scatter signal from bacteria depends on its shape and on the range of scatter angles detected. As bacterial populations are morphologically heterogeneous, we will investigate to which extend results may vary according to the composition (possibly taking into account age and density) of reference populations. Furthermore, a study will be performed in order to analyse whether a change in the bacterial culture (due to viral infection, specified stress,..) could be correlated with a change in size and granularity. Finally, a possible relationship between the side scatter (SSC) and bacterial morphology will be investigated. Visual evaluation is to be performed with phase contrast microscopy. All the changes will be quantified by the use of calibrated microspheres of known diameter size (0.1, 0.2, 0.5, 1, 2, 5 and 10 µm diameter) and a calibration curve of the mean channel versus the size will be established. If there is heterogeneity of the bacterial size, it should be reflected by a high variation coefficient.

Below are two tables taken from Environmental Monitoring of Bacteria that display the different types of fluorescent dyes that may be used for flow cytometry labeling.

Fluorochrome	Target molecules
Fluorescein and tetramethylrhodamine	General fluorescent labels, via isothiocyanate group; e.g. total cell protein, antisera, oligonucleotides
Phycoerythrin	Conjugated to protein, usually for immunofluorescence
Ethidium Bromide	Double-stranded nucleic acid, often with mithramycin
Propidium Iodide	Labeling double-stranded nucleic acid
Mithramycin	G/C rich DNA, often used with ethidium bromide
Chromomycin A3	G/C rich DNA
Hoechst 33342 or 33258	A/T rich DNA
4',6-Diamidino-2-phenylindole	Nucleic acid dye
SYBR Green I	Nucleic acid
YOYO-1, YOPRO-1	Cyanine dye-based nucleic acid
PicoGreen	Double-stranded nucleic acid

Table 5.1 : General fluorochromes used to label bacteria for flow cytometry.

Fluorochrome	Cell function measured
Rhodamine 123	Membrane potential
Dihexylocarbocyanine dyes	Membrane potential (several forms exist)
Fluorescein diacetate	Enzyme activity, membrane integrity
Carboxyfluorescein diacetate	Enzyme activity, membrane integrity
Bis-carboxyethyl-carboxyfluorescein acetoxymethyl ester	Enzyme activity, membrane integrity
Calcein acetoxymethyl ester	Enzyme activity, membrane integrity
Chemchrome B	Enzyme activity, membrane integrity
Cyanoditolyl tetrazolium chloride	Respiratory activity
Ethidium Bromide	Double-stranded nucleic acid
Propidium Iodide	Double-stranded nucleic acid
Bac Light	Commercial kit, nucleic acid
Oxonol dyes Calcofluor White Popro	Accumulate in dead cell membranes Nucleic acid Nucleic acid
SYTOX Green	Nucleic acid

Table 5.2 : Fluorescent probes used to assess bacterial viability by flow cytometry.

Flow cytometry can produce a wide variety of data types, with different fluorochromes available to do a variety of experiments (Tables 5.1 and 5.2). Investigations include many different environmental samples, in particular aquatic samples, but flow cytometry is also applicable to other fields as shown in Table 5.3.

Author/Year/Journal	Study Preformed	Sample Type	Dye/Method Used
Andreatta et al. 2000 Cytometry	Image analysis to detect subgroups	Heterotrophic Bacterioplankton	DAPI/ TransFloroSpheres
Bernard et al. 2000 Microb. Ecol.	Relationship cell size, productivity, and genetic diversity	Aquatic Mediterranean	SYTO/Cell Sorting
Bernard et al. 2001 Cytometry	Genetic diversity of viable and active bacteria	Seawater cultures	DVC/ CTC/Cell Sorting
Bouvier et al. 2001 Cytometry	Estimate Bacteria Biovolume	Bacterial strains of aquatic env.	DAPI/ Light Scattering
Button et al. 2001 AEM	Determination of DNA Content	Seawater/marine org.	DAPI/Triton X-100
Davey et al. 1999 Cytometry	Identification of Microorganisms	*B.subtilis, E.coli, M. luteus*	TinopalCBS-X/SYTO 17/Oxonal V/ TO-PRO-3
Dubelaar et. al. 1999 Cytometry	*In situ* analysis of marine and fresh water	Marine and fresh water	CytoBuoy: a wireless Flow cytometer
Edwards et al. 1999 Cytometry	Rapid sequential flow cytometry	Chinese hamster ovary cells	GFP/hydroethidine/Plug flow
Gasol et al. 1999 AEM	Significance of size and nucelic acid content	Planktonic bacteria	Live/Dead kit/ epiflourescence microscopy
Gregori et al. 2001 AEM	Resolution of viable mebrane-compromised bacteria	Fresh and marine water samples	Live/Dead SYBR Green I & II and PI
Huage et al. 1999 Cytometry	Bacterial genome fingerprinting	*S.aureus* and ?-phage	T0T0-1/ PFGE
Jacoberger et al. 1999 Cytometry	Cell-Cycle-regulated gene expression	Human prostate cancer cell line	FITC-anti-cyclin B1
Larson et al. 2000 Cytometry	Rapid DNA fingerprinting of Pathogens	*S. aureus, E.coli*	PFGE / PicoGreen dye
Robertson et al. 1998 AEM.	Determination Biomass of small bacteria in low concentrations in mixed species	*C.oliotrophus, Marinobacter sp., P.diminuta*	DAPI/Triton X-100
Simon et al. 1995 AEM	FISH using rRNA probes to identify Small Phytoplankton	*Phylas Chlorophyta, Heteroconta, Dinophyta, and Cryptophyta*	
Schmid et al. 1999 Cytometry	Live cell growth and phenotype in population with low viability	Molt-4f t-cell leukemia	7-AAD/ Leu-3a FITC
Zubov et al. 1999 AEM	Determination of total protein content of bacterial cells	Seawater sample isolates	SYPRO

Table 5.3: Selected Studies Exhibiting Multiple Uses Of Flow Cytometry

5.2.4. Assessment of viability, survival, membrane potential and permeability of the MELISSA strains

In the last few years, various studies for the rapid assessment of bacterial viability and survival have been undertaken. In addition, numerous applications of flow cytometry to analyse drug susceptibility of eukaryotes and prokaryotes have been reported. Efficient dyes for the assessment of viability and survival are **Rhodamine 123, oxonol, and propidium iodide**. The signals from these fluorochromes detect changes in membrane potential or envelope integrity of the cells. The level of accumulation of Rhodamine 123 and oxonol inside the cell depends on its membrane potential or the difference in charge between both sides of the plasma membrane. A study will be performed in order to find a rapid method to assess bacterial survival and viability among the bacterial populations. This is based on the hypothesis that viral contamination or stress may disrupt or alter the outer membrane and cell membrane in bacteria, resulting in changes in the integrity of cell envelopes and membrane potential. **Rhodamine 123** is a cationic lipophilic dye (accumulated cytosolically by cells with an inside negative transmembrane

electrochemical potential) that has been used extensively to study bacterial viability. **Oxonols** [i.e. bis-oxonol DiBaC(4)3] are anionic lipophilic dyes which, unlike Rhodamine 123, are not extensively accumulated cytosolically by cells with an inside negative transmembrane potential, and which have also been used to assess bacterial viability. Therefore, their fluorescence response is opposite that of cationic Rhodamine 123. When membrane potential increases, the fluorescence response of Rhodamine increases, but the fluorescence response of oxonol decreases. Conversely, when membrane potential decreases, Rhodamine 123 fluorescence decreases but oxonol fluorescence increases.

Propidium iodide (a phenanthridinium dye) is a membrane-impermeant dye that stains by intercalating into nucleic acid molecules stains nucleic acid molecules. It binds RNA and DNA and exhibits no sequence specificity (one dye per 4-5 base pairs of DNA). On binding to nucleic acids, the fluorescence yield is enhanced ~ 25-fold, and the excitation maxima is shifted ~30-40 nm to the red and the emission maxima is shifted ~15 nm to the blue. In the DNA-bound form, propidium iodide excites at 536 nm and fluoresces at 617 nm. Propidium Iodide is cell membrane impermeant dye and can be used for evaluating viable cells (will stain negative). It is also useful in double-label experiments to detect DNA and a fluorescein-labeled antigen simultaneously.

The overall set-up of the viability protocol based on Rhodamine 123, bis-oxonol DiBaC(4)3, and propidium iodide stainings is determined by the following steps that will each have to be optimised :

- In order to permeabilise the bacterial outer membrane, EGTA (at a final concentration of 1 mM) will be added to an overnight culture (or maybe more depending on the length of the cell cycle of the bacterial populations) previously diluted 1/1000 in NaCl 0.9%.
- Rhodamine 123 will be added to a final concentration of around 0.2 µg/ml (concentration to be optimised) from a stock solution in ethanol.
- Propidium iodide will be added to a final concentration of around 10 µg/ml (concentration to be optimised).
- Oxonol (around 4 µl of 250 µM solution of oxonol in ethanol) will be added to 1ml of bacterial suspension and incubated for a few minutes (between 2 and 5 minutes) at room temperature.

Remark: Oxonol and Rhodamine 123 staining at different culture times will have to be determined in order to check whether Rhodamine 123 and oxonol are taken up at different rates during the exponential and stationary phases. A progressive decline in Rhodamine uptake is usually observed during the growth phase. Bacteria showing high Rhodamine 123 fluorescence should exhibit little or no fluorescence with oxonol. Because the fluorescence intensity of Rhodamine 123-stained populations is highly dependent upon their physiological state, standardisation of the inoculum is an important factor that has to be chosen for flow cytometrical experiments.

5.2.5. Assessment of the respiratory activity of the MELISSA strains.

Cell-specific assays to detect respiratory activity of bacteria have been developed based on the use of different tetrazolium salts. Tetrazolium dyes are reduced from a colourless complex to a brightly coloured, intracellular, formazan precipitate by components of the

electron transport system and/or a variety of dehydrogenase enzymes present in active bacterial cells. Since electron transport is directly related to cellular energy metabolism in respiring cells, the ability of cells to reduce tetrazolium compounds can be considered an indicator of bacterial activity. A variant approach is the use of the redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC). CTC is reduced by bacteria to a water-insoluble, red fluorescent formazan product. It allows the quantification of the metabolic activity of bacteria under both aerobic and different anaerobic conditions. CTC is commonly used in microbial ecology, for both aquatic and terrestrial systems. Applications include drinking water, biofilms, lake and sea-water and sediments.

5.2.6. Assessment of the (metabolic) esterase activity of the MELISSA strains.

Detection of esterase activity is measured using lipophilic, uncharged and non-fluorescent fluorogenic substrates. Once within active cells, the substrate is cleaved by non-specific esterases releasing a polar fluorescent product (fluorescein or fluorescein derivatives) retained inside cells having an intact membrane. Esterases are present in all living organisms, and these enzymes can be used to provide information on the metabolic state of bacterial cells. Although enzyme synthesis requires energy, the enzyme-substrate reaction does not, and this assay alone should be considered energy independent. However, dead or dying cells with damaged membranes rapidly leak the dye, even if they retain some residual esterase activity. Consequently, fluorogenic substrates for esterases often serve as activity and cell integrity probes that measure both enzymatic activity, which is required to activate their fluorescence, and cell-membrane integrity, which is required for intracellular retention of their fluorescent products.
Fluorescein diacetate (FDA) is known to give weak fluorescence signals, since fluorescein is poorly retained inside the cells. In contrast, hydrophobic FDA derivatives are cleaved into hydrophilic products that are retained more efficiently inside cells with an intact membrane. Among these, acetoxymethyl ester (calcein-AM) was shown ineffective to label different species, with the exception of Staphyllococcus aureus.

5.2.7. Use of flow cytometry to check DNA content changes in the MELISSA strains.

Flow cytometry is uniquely suited for cell cycle analysis, since information about different cell cycle stages can be obtained without the need for synchronization of the cell culture. Also, flow cytometry offers the advantage that it can be applied to organisms whose genetics and physiology are largely unknown; as long as a DNA-specific stain can be made to enter the cells and reach the nucleic acid, flow cytometry can be performed on most species from which single cell suspensions can be obtained.

In cell cycle analyses of eukaryotic organisms, flow cytometry is commonly employed, and a variety of instrument configurations, light sources and stains are in use. In contrast, flow cytometric analysis of bacterial cells is not widely used. Previously, the reason for this was that the resolution and sensitivity of conventional flow cytometers was near or below the detection limit for bacterial analysis; the DNA content of a bacterial cell is about 1,000) fold less than that of a human cell, and the cell mass difference is also considerable.

With the development of a flow cytometer with a different configuration, flow cytometry of bacteria became feasible and several laboratories have used this instrument for

bacterial cell cycle analysis. Unique information has been gathered, including measurements of lengths of different cell cycle periods in various *Escherichia coli* strains subjected to different growth conditions, the degree of coordination of initiation at multiple origins of DNA replication, the variation in the DNA content and cell size distributions of bacterial populations at different stages of growth in batch cultures as well as other cell cycle studies. Characterisation of DNA replication and cell division patterns in different mutant strains has been carried out, as well as characterisation of strains in which chromosome replication is controlled by integrated plasmid replicons. Other uses include studies of antibiotic effects as well as analysis of clinical material.

The quantitative measurement of the DNA content became possible by specific fluorescent dyes which bound stoichio-metrically to the double helix of nuclear DNA. The fluorescence inside the cell-nucleus can easily be detected using flow cytometry. Thus, the amount of DNA for cell populations can be studied in regard to i) the cell cycle and ii) the ploidy level.

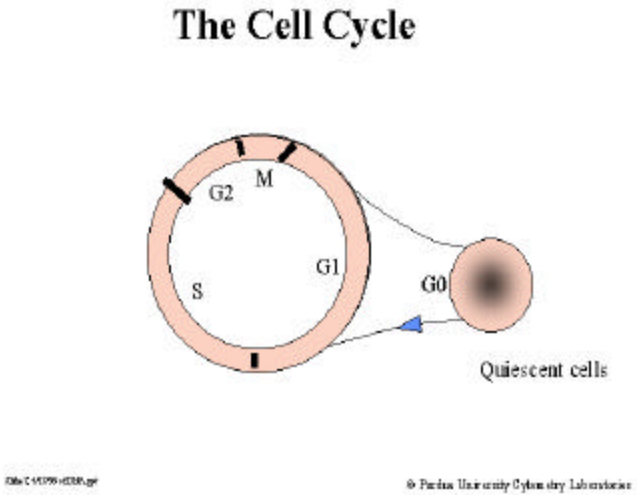

Figure 5.3 : The cell cycle.

In a growing population, the amount of DNA per cell is correlated to certain cell cycle phases of chromosomal replication. Three different phases can be distinguished: G1, S, G2M (G1: normal DNA content = 2c, S: DNA synthesis, G2M: double DNA content = 4c and mitosis) (Fig. 5.3).

The peaks of the G1 and G2M compartments in a DNA histogram show Gaussian distributions (Fig. 5.4. gives a typical DNA content profile obtained by flow cytometry on a bacterial population). Different mathematical models are available to integrate peak areas in order to determine cell counts (in percentiles) of the different cell cycle phases.

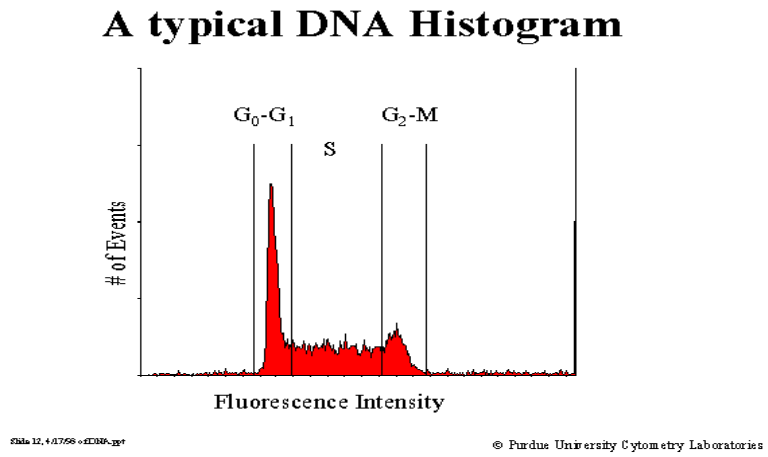

Figure 5.4 : DNA profile of a bacterial population.

5.2.8 Preparation of samples to analyse DNA content from bacterial cultures

Retention of fully replicated chromosomes . To obtain bacteria that contain only fully replicated chromosomes, different substances such as rifampicin (around 150 µg/ml) or cephalexin (around 10 µg/ml) will have to be added to the culture grown to an optical density of 0.1 to 0.3. The molecular mechanism of rifampicin is an inhibition of bacterial RNA polymerase that forms a complex with rifampicin. Rifampicin prevents the ribosomes from binding to messenger RNA, and thus proteins are not produced.
Flow cytometry is useful in order to study the DNA distribution of an exponentially *E. coli* growing culture (Fig. 5.5). Note that the DNA distribution resembles that of the age. distribution, there are more cells with the low DNA content corresponding to a little more than 2 fully replicated chromosomes, than cells with twice the amount of DNA. Fig. 12B shows the DNA distribution of a sample from the same culture incubated for 3 hours in the presence of antibiotics inhibiting protein synthesis and cell division. The DNA now occurs in discrete peaks, most of the cells have 4 fully replicated chromosomes. The number of fully replicated chromosomes corresponds to the number of origins present in the cell at the time of drug addition. Cultures will have to be preincubated for around 3 hours (time to optimise) in the presence of rifampicin and cephalexin in order to allow complete runout of replication, such that all cells contain only fully replicated chromosomes.

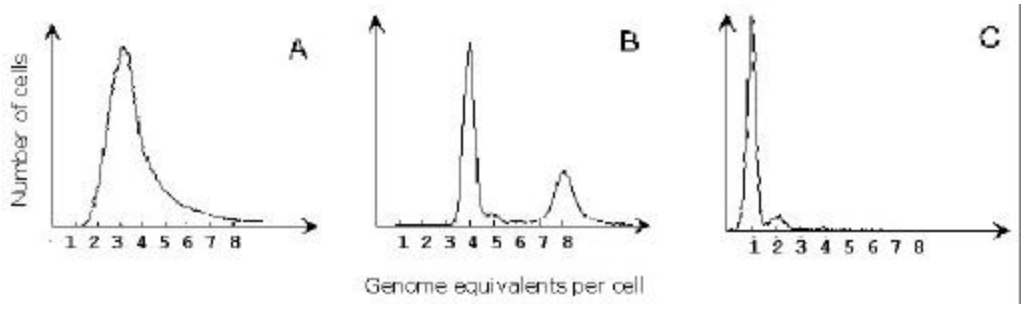

Figure 5.5.: Flow cytometric DNA distributions. A sample from an exponentially growing culture of *E. coli* (A), the same culture incubated for 5 hrs with rifampicin and cephalexin (B), and a 30°C culture of a *dnaA46* mutant incubated at 42°C for 2 hrs (C)

DNA staining. Samples will be chilled in 1.5 ml of chilled Tris buffer (Tris 10 mM buffer + 1 mM EDTA) pelleted and washed in Tris buffer, pelleted again, and resuspended in the same buffer before fixation in 70%-80% cold ethanol during vortexing.Different DNA stains will have then to be compared and the optimal concentration range will have to be determined from dilution experiments:

- Propidium iodide (around 50 µg/ml)
- Mithramycin A (around 200 µg/ml)
- 7AAD (around 50 µg/ml)
- Ethidium bromide (around 40 µg/ml)

RNAse treatment. Since DNA stains (propidium iodide, 7AAD) generally bind double strain RNA, the sample had normally to be treated with RNAse for DNA measurements to be correct. Otherwise, the fluorescence from RNA bound dye molecules may broaden the DNA distributions. However, some authors do not include any RNAse treatment. A comparison between the presence or the absence of RNAse treatment will have to be performed.

Coefficient of variation. The coefficient of variation (CV) of a peak area is given by the term standard deviation divided by mean channel number in percent. A low CV value indicates a high resolution analysis due to a very homogenous DNA staining and excellent instrument performance in detecting the fluorescent signal. With the EPICS XL instruments, very low CV values (lower than 1,5 %) can be obtained for various cell samples. Obtained CV values will be used to compare staining procedures and to determine required concentrations of rifampicin and cephalexin.

5.3. Detection of viral contamination of the MELISSA strains by flow cytometry

5.3.1. Flow cytometric detection of viruses alone.

It has been considered previously nearly impossible to detect virus particles using conventional flow cytometry because of their small size. However, following the introduction of new nucleic acid staining dyes, which emit in the green with a high fluorescence yield after excitation at 488 nm, the limit of detection was improved considerably. Recently, Marie et al. (1999) reported the successful use of a standard flow cytometer with a low-power argon-ion laser to detect and enumerate viruses in sea water after staining with the nucleic acid specific dye SYBR Green I. The investigators developed their methodology using the lytic virus PpV-01, which infects the marine unicellular alga *Phaeocystis pouchetii,* and tested the assay on natural samples from various oceanic locations. It is still unknown, however, if this method can be applied generally to all viruses, where the detection limit of detection lies, and whether there is a linear relationship between the fluorescence signal and viral genome size. Brussaard and colleagues showed that viruses from different families (Baculoviridae, Herpesviridae, Myoviridae, Phycodnaviridae, Picornaviridae, Podoviridae, Retroviridae, and Siphoviridae) could be detected using flow cytometry after staining with SYBR Green I. A linear correlation was not found between green fluorescence intensity and genome size (7.4-300 kb). The fluorescence signal of the RNA viruses tested, having genome sizes between 7.4 and 14.5 kb, could not be distinguished easily from background using this staining protocol and a standard air-cooled low-power flow cytometer.

Virus abbreviation	Name	Family	Type	Particle size (nm)	Genome size (kbp)	Tail (nm)	Enveloped	Reference
Bac	Insect Autographa californica polyhedrosis virus ACNPV virus Bac	Baculoviridae	Circular ds DNA	300 x 50	130	No	Yes	Fields et al., 1996
CeV-B01	Algal Chrysochromulina ericina, virus CeV	Phycodnaviridae	ds DNA	140		No	No	Bratbak, unpublished data
HIV-1	Human immunodeficiency, virus HIV 1	Retroviridae	Dimeric ss (+) strands DNA	110	9.2	No	Yes	Fields et al., 1996
HSV-1	Human Herpes simplex, virus HSV type 1	Herpesviridae	Linear ds DNA	100-110	152	No	Yes	Fields et al., 1996
ISA-V	Infectious salmon anemia, virus ISAV	Orthomyxoviridae	Ss RNA	100-130	14.5	No	Yes	Mjaaland et al., 1997
Lambda	Coliphage Lambda	Siphoviridae	Linear ds DNA	55	48.5	150 x 8	No	Murphy et al., 1995
MpV-SPI	Algal Micromonas pusilla, virus MpVSPI	Phycodnaviridae	Linear ds DNA	115	200	No	No	Suttle, personal communication
MpVUF10-38	Algal Micromonas pusilla, virus MpVUFl0-38	Phycodnaviridae	ds DNA	100-140		No	No	Sahlsten, personal communication
PBVC-1	Paramecium burscaria-Chlorella virus PBCV-1	Phycodnaviridae	Linear ds DNA	160-190	300	No	No	Van Etten et al, 1985
PoV-B01	Algal Pyramimonus orientalis, virus PoV	Phycodnaviridae	ds DNA	200		No	No	Bratbak, unpublished Data
PpV-01	Algal Phaeocystis pouchetii, virus Ppv-01	Phycodnaviridae	ds DNA	130-160		No	No	Jacobsen et al, 1996
PV-1	Human Poliovirus PV	Picomaviridae	Ss (+) strand RNA	30	7.4	No	No	Fields et al., 1996
S-PM2	Cyanobacterial Synechococcus sp., virus S-PM2	Myoviridae	Linear ds DNA	90	90-100	165 x 20	No	Wilson et al, 1993
T2	Bacteriophage T2	Myoviridae	Circular ds DNA	85 x 110	158-205	110 x 85	No	Murphy et al., 1995
T4	Bacteriophage T4	Myoviridae	Circular ds DNA	78 x 111	171-214	113 x 16	No	Murphy et al., 1995
T7	Bacteriophage T7	Podoviridae	Linear ds DNA	60	39.9	17 x 8	No	Murphy et al., 1995

Table 5.4: Characteristics of the viruses used in Brussaard *et al.* study (2000).

5.3.2. Flow cytometric detection of viral-infected phytoplankton cells.

Flow cytometry can detect viral antigens either within or on the surface of infected cells. It can rapidly detect and quantify virus-infected cells using antibodies that specifically recognise surface or internal antigens. Based on the potential of flow cytometry for multiparametric analysis, there are two key advantages to its use in studying viral infection : (i) its ability to analyse several parameters in single -infected cells at the same time and (ii) its ability not only to detect but also quantify infected cells. These parameters may be related to particular components or events of the infected cell or components (proteins or nucleic acids) of the virus. For this reason, flow cytometry is a powerful tool to characterise the mechanisms of viral infection. Furthermore, flow cytometry allows simultaneous detection of several viruses in a sample using (i) antibodies to different viral antigens conjugated to different fluorochromes or (ii) specific viral antibodies conjugated to latex particles of different sizes. The presence of different viral antigens is detected by differences in the forward-scattered light as a consequence of the different sized particle used for each antibody. For example, one can simultaneously detect cucumber mosaic, tomato and potato viruses by using 3-, 6- and 10-µm diameter latex particles, respectively. Although this method was aimed at the detection of plant viruses, its basis could be applied to the detection of bacterial viruses.

Current working hypotheses suggest that viruses can be important regulating factors in microbial ecosystems. For instance, lytic bacteriophages may directly control the population dynamics through cell lysis. In addition, phage-mediated gene transfer may alter the genetic composition of the microbial community. Recent research in aquatic microbiology has concentrated on the detection and enumeration of algal viruses. However, detailed information on the direct effect of a viral infection on phytoplankton cells is still lacking. Waters and Chan (1982) and Suttle et al. (1990) showed that viral infection may lead to a reduction in chlorophyll fluorescence and primary production of the algal species, but these data do not provide any information about individual cells within the algal population. The detection of viruslike particles within algal cells using transmission electron microscopy (TEM) provides only information about the final stages of the lytic cycle when mature viral particles are visible. Furthermore, TEM analyses are time consuming. Flow cytometry (FCM) allows a rapid and precise analysis of the characteristics of individual cells and is a tool with great potential for the study of changes in cellular parameters of virus infected and noninfected algal populations. Because of *de novo* synthesis of viral DNA and possible digestion of host DNA, the cellular DNA content of virus infected and noninfected phytoplankton cells may be expected to be different. The use of FCM in combination with nucleic acid specific fluorescent dyes allows quantification of cellular DNA. Recently, FCM has also been successfully applied to enumerate algal viruses, making flow cytometry a potentially useful tool for studies on basic virus-host cell interactions.

Brussaard and colleagues (1999) followed the cell characteristics of two phytoplankton species, *Micromonas pusilla* (Butscher) Manton et Parke and *Phaeocystis pouchetii* (Hariot) Lagerheim, during viral infection using flow cytometry. Distinct differences between noninfected and infected cultures were detected in the forward scatter intensities for both algal species. Changes in side scatter signals on viral infection were found only for *P. pouchetii*. Chlorophyll red fluorescence intensity per cell decreased gradually over time in the infected cultures. DNA analyses were performed using the nucleic acid–specific fluorescent dye SYBR Green I. Shortly after infection the fraction of algal cells

with more than one genome equivalent increased for both species because of the replication of viral DNA in the infected cells.

Recently, Brussaard et al (2001) used two flow cytometric assays using physiological probes on the phytoplankton species *Phaeocystis pouchetii* and *Micromonas pusilla* to examine the assays' utility in detecting viral infections. Dead cells were detected using the membrane impermeant nucleic-acid dye SYTOX-Green, which stains algal cells that have lost their membrane integrity. Live cells were detected using the membrane permeant dye Calcein-AM, which is hydrolyzed by intracellular esterases into a green fluorescent charged form. They found that both assays are easy to use and reproducible and both dyes cann be used as markers of the viability of individual phytoplankton cells following infection by viruses.

5.4. Towards the ideal solution: coupling PCR to flow cytometry ?

The emergence of PCR and RT-PCR techniques has allowed the highly sensitive detection of specific viral nucleic acids (DNA or RNA) in virus-infected cells. These methods are indeed the most sensitive for the detection and characterisation of viral genomes, especially in the case of rare target viral sequences. However, the association between the viral nucleic acid and an individual cell is lost, and therefore no information about productively infected cell populations is obtained by this method. Flow cytometry analysis of fluorescent in situ hybridisation in cell suspension overcomes this problem, since this assay can be coupled with simultaneous cell phenotyping.

For example, flow cytometry detection of in situ hybridisation has been used to analyse rare virus-producing cells in peripheral blood samples. HIV-1 RNA in infected cell lines was detected by fixation of the cells in suspension, hybridisation with HIV-1 genomic probes labelled with digoxigenin-11-dUTP, detection with fluorescent anti-digoxigenin antibody, and flow cytometry analysis of the fluorescence signals thus generated. In this way, one could detect CMV antigen with immunoenzymatic labeling after 4 days, whereas CMV-DNA was detectable by PCR (coupled to flow cytometry detection) using *in situ* hybridisation just 4 hours after infection of T-lymphoblastoid cells.

Using this method, the dentification of specific viral DNA or RNA should permit latency studies in viral infections through the identification of specific cells actively replicating the infective virus or cells that harbour the virus in a latent state, acting as a reservoir for infection (with the possibility of phenotyping cells by flow cytometry). Furthermore, flow cytometry permits these cells to be sorted for the characterisation of latency and reactivation mechanisms.

Recently, a very sensitive and powerful PCR-driven *in situ* hybridisation assay has been developed. This method combines the sensitivity of PCR with the specificity of *in situ* hybridisation, allowing rapid and reproducible detection of single-copy proviral DNA or low-abundance viral mRNA in subsets of cells in suspension. This assay employs PCR- or RT-PCR-driven in situ amplification of viral sequences in fixed cells in suspension with sequence-specific primers and digoxigenin-linked dUTP. The product DNA is hybridised with a fluorescein-labelled oligonucleotide probe, and the cell suspension is then analysed by flow cytometry. The sensitivity and specificity of this technique revealed a linear relationship for the detection of a single copy of intracellular proviral DNA over a wide range of virally infected cell concentrations.

The flow cytometry nucleic acid detection techniques have similar sensitivity to conventional PCR, but with the added benefits derived from expression analysis in individual cells (in conventional PCR, nucleic acid expression is not analysed independently in each cell). Multiparametric analysis of infected cells allows the detection of single-copy proviral DNA or low-abundance viral mRNA in specific subsets of cells that can be phenotyped at the same time. Moreover, flow cytometry is a very useful tool to study the mechanism of viral latency by association of different stages of the virus cycle and disease progression with the location of the virus in specific cell populations. This can be achieved by using probes to specific mRNAs related to different viral replication stages. Knowledge of cell populations in which the virus is either replicating or in a latent state has important implications for our understanding of virus replication and progression of the infection and hence for treatment. Furthermore, it should be possible to sort these cells for further analysis. Double staining of viral nucleic acids together with viral proteins or surface markers is also possible. In comparison with the detection of the PCR-driven in situ hybridisation by fluorescence microscopy, in which a large number of microscopic fields must be studied, flow cytometry allows the analysis of thousands of cells in a few seconds. The speed and automation of these assays make them optimal for the rapid diagnosis of viral infections.

FCM can also facilitate PCR amplification and cloning of 16S rDNA based on the specific light scattering properties. This is accomplished by sorting cells previously hybridized and probed via rRNA fluorescent probes *in situ*. These "probe positive" cells can then be sorted directly onto microscope slides. Such a technique can serve to enrich for certain cells with particular characteristics, which can then be followed by PCR amplification of 16S rDNA. This DNA can subsequently be cloned for further analysis (e.g. sequence analysis). This technique allows the observer to rapidly screen for cells with certain characteristics while by-passing culturing. In the future, this type of procedure may be further developed to investigate the presence of functional genes and perform quantitative PCR analysis of mRNA on organisms that cannot be cultured.

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Addendum. Evaluation table of methodologies in the axenicity study

Method		Mass (kg)	Cost (€)	Cost/ sample	Running time	Spacialization
PCR	Consumables (enzymes, chemical products, tubes, ..)(*) Water consumption (buffers, washing, ..) (*) Other lab material (automatic pipettes, racks, ..) Equipment: - storage (small fridge / -20°C freezer / ..) - microcentrifuge - PCR apparatus - electrophoresis (buffer tanks, power supply, ..) - data acquisition (UV box, camera, ..) - data analysis (laptop PC, ..) SUBTOTAL	6 30 2 40 (s) 5 (s) 8 3 6 3.5 (s)	2000 - 1200 500 1200 2600 2000 2000 2000 13500	(excluding equipment costs and assuming a minimum throughput of 300 samples) 4 € / sample	From start to finish, for 12 samples: 6 h breakdown: DNA prep: 2 h PCR: 2 h Electrophoresis: 1 h Data A&A: 1 h	Use of nano-technology will reduce water consumption. DNA prep., PCR, electro-phoresis, and data A&A can be done on micro-scale, reducing mass and increasing speed.
MALDI-TOF	Consumables (enzymes, chemical products, probe, eppendorf tubes) Other lab material: - Automatic pipettes - Racks Equipment : - storage (small fridge / -20°C freezer / ..) - microcentrifuge - Maldi-tof apparatus - Data acquisition and analysis (2 laptop PC) SUBTOTAL	2 1 1 40 (s) 0.5 (s) 130 (s) 7 (s)	2000 1000 200 500 200 196000 4000 203900	(excluding equipment costs and assuming a minimum throughput of 100 samples) 4 € / sample	From start to finish, for 150 samples: 1h30 breakdown: bacterial prep: 1h Maldi-tof: 20min. Data A&A: 10min.	High through put setup with automated data A&A and preparation and introduction of the different samples in the MALDI-TOF system (Massprep ™, Micromass Inc.). Miniaturization and on-line monitoring are possible.
FCM	EPICS XL flow cytometer bench apparatus compressor computer screen Data acquisition system and data analyser with various software (Windows, EPICS XL softwares : System II + EXPO 32) Chemical products: buffers, beads, dyes, fluorochromes, etc. Other lab material: tubes, automatic pipettes, tips, racks, … Small equipment: - storage (small fridge) - microcentrifuge SUBTOTAL	84.8 54.4 14.5 22 50 2 2 40 (s) 5 (s)	75000 3000 1500 1500 500 1200 82700	(excluding equipment costs) 5 to 20 € / sample (depending on the type of analysis performed)	From start to finish for 10 samples depending on the type of analysis (from 2 to 5 hours) Preparation : 0.5 to 2 h FCM : 1 to 2 h Data A&A : 0.5 to 1 h	The current versions of flow cytometers available on the market are too sensitive to be sent into space.
HGT	Consumables (enzymes, chemical products, tubes, plates) Water consumption Other lab equipment (pipettes, racks,..) Equipment: - storage (small fridge/-20°C freezer/..) - microcentrifuge - PCR apparatus - electrophoresis (buffer tanks, power supply, ..) - data acquisition (UV box, camera, ..) - data analysis (laptop PC, ..) - spectrophotometer SUBTOTAL	10 80 2 (s) 40 (s) 5 (s) 8 3 6 3.5 (s) 2	3500 - 1200 500 1200 2600 2000 2000 2000 2500 17500	(excluding equipment costs and assuming a minimum throughput of 200 samples) 1 to 4 € / sample (depends on the extent of PCR-based analysis)	For 12 samples Conjugations: growth ref strain: 1 d mating: 1 d selection: 1 d Detection of free DNA/plasmids/phages: DNA prep: 2 h PCR: 2 h Electrophoresis: 1 h data A&A:1 h	If bioassays can be used with reference bacteria, all equipment can be elliminated. Main costs will be the consumables (2000 €). Main mass will be water consumption (50 kg).

(s) shared; (*) estimated consumption per year; A&A: acquisition and analysis