MELiSSA

TECHNICAL NOTE

Memorandum of Understanding TOS-MCT/2002/3161/In/CL

SCK • CEN

UMH
UNIVERSITE DE MONS-HAINAUT

ULg
UNIVERSITÉ de Liège

TECHNICAL NOTE: 70.12

PART A: PROPOSED FUTURE WORK FOR LONG-TIME AXENICITY STUDY

prepared by/*préparé par*	L. Hendrickx, R. Wattiez, S. Baatout, A. Wilmotte, P. Janssen, M. Mergeay
reference/*réference*	15680/01/NL/ND
issue/*édition*	1
revision/*révision*	0
date of issue/*date d'édition*	
status/*état*	Draft
Document type/*type dedocument*	Technical Note
Distribution/*distribution*	C. Paillé, N. Dorval, R. Wattiez, A. Wilmotte, M. Mergeay, S. Baatout, L. Hendrickx, P. Janssen

CONFIDENTIAL DOCUMENT

APPROVAL

Title *titre*		issue *issue*	1 revision 0 *revision*
author *auteur*	L. Hendrickx, S. Baatout, R. Wattiez, A. Wilmotte, P. Janssen, M. Mergeay	date *date*	27/08/ 2004
approved by *approuvé by*	Christel Paillé	date *date*	23/11/ 2005

CHANGE LOG

reason for change */raison du changement*	issue/*issue*	revision/*revision*	date/*date*
	1	1	

CHANGE RECORD

Issue: 1 Revision: 1

reason for change/*raison du changement*	page(s)/*page(s)*	paragraph(s)/*paragraph(s)*

TABLE OF CONTENTS

1 INTRODUCTION 2

2 MELGEN, THE TOOLS 3

3 FUTURE DIRECTIONS 10

3.1 FLUORESCENT PROBES 10
3.1.1 Introduction 10
3.1.2 Using fluorescent probes 10
3.1.3 The experimental strategy 10
3.2 FLUORESCENT ANTIBODIES 11
3.2.1 Introduction 11
3.2.2 Using fluorescent antibodies 12
3.2.3 The experimental strategy 12
3.3 PNA PROBES 13
3.3.1 Introduction 13
3.3.2 Using PNA probes 13
3.3.3 The experimental strategy 13
3.4 PRECISE ESTIMATION OF THE LIMITS OF DETECTION OF AXENICITY BY FLOW CYTOMETRY IN MELGEN BACTERIAL STRAINS 14
3.4.1 Comparison of bacterial counting by flow cytometry, plating and optical density on MELGEN bacterial strains 14
3.4.2 Comparison of cell size estimation by microscopy and flow cytometry 14

ABREVIATIONS

BHR	Broad-Host Range
dNTPs	Deoxynuleotide triphosphates
EDTA	Ethylene-DiamineTetraAcetic acid
EtBr	Ethidium Bromide (2,7-diamino-10-ethyl-9-phenyl-phenanthridium bromide)
FCM	Flow CytoMetry
FISH	Fluorescence in situ Hybridisation
FL	fluorescence line
FS	forward scatter
HPLC	High-Pressure (or High-Performance) Liquid Chromatography
HPCV	half peak CV
ICM-MS	Intact cell MALDI_TOF MS
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time-of-Flight
MESF	molecules of equivalent soluble fluorochrome
MFI	mean fluorescence intensity
MS	Mass spectrometry
OD	Optical Density
PCR	Polymerase Chain Reaction
PNA	Peptide nucleic acid
rDNA	rRNA encoding DNA
SAPD	Surface Accessible Proteins Detection
SCM-MS	Supernatant MALDI-TOF MS
SS	side scatter
TCA	TriChloroacetic Acid
UV	Ultra Violet

1 INTRODUCTION

In MELGEN, 3 ways of detecting contaminants were evaluated: flow cytometry, intact cell mass spectrometry and PCR. To insure the stability of the bioprocesses in each compartment the avoidance of contamination (including the transfer of genetic material) shall be insured and controlled by early tracking contamination events. Contaminations may result into metabolism change, production of toxic compounds, strengthening the functioning of the loop and rendering *Arthrospira* and *Rhodospirillum* biomasses risky to be consumed. Similar techniques, as the ones selected to investigate the microbial response to stressors, were set-up and validated on *R. rubrum*, *A. platensis* and *R. metallidurans* (robust strain used as model). Cultural assays to investigate the possible capture of plasmids or bacteriophages by the strains of interest were set-up and validated. Preliminary experiments demonstrated the *R. rubrum* ability to exchange BHR plasmids at high frequency (10^{-2}-10^{-3}) in bi- and triparental matings.

2 MELGEN, THE TOOLS

Cultivation: Inoculation on solid media or in broth is the most classical method to detect the presence of pathogens in the clinical applications. Since Pasteur and Koch, many culture media and a great number of tests were developed and specifically enable the microbiologists to identify the micro organisms that grow (Hobson *et al.*, 1996).

Microscopic techniques (light, confocal, electron...) are useful when the organisms have different morphologies. For example, the presence of bacterial contaminants in *Arthrospira* cultures could be detected after staining with DAPI. Using fluorescence microscopy, the *Arthrospira* appear red due to their autofluorescence, whereas the bacteria appear dark blue (stained DNA) and the cell debris that have a similar size to the bacteria appear light blue. The confocal microscopy has been used to study the interactions of bacteria with food, and show their spatial localisation (Takeuchi & Frank, 2001).

Flow cytometry: This method has been applied in conjunction with viability markers for rapid counting of yeast, mould and bacterial cells in food products (Laplace-Builhe *et al.*, 1993), and in milk (Gunasekera *et al.,* 2000). A recent development is using a laser-induced fluorescence coupled with flow cytometry to detect single contaminants in real time (Johnson *et al.*, 2001).

Flow cytometry is a method for quantitating components or structural features of cells primarily by optical means. Although it makes measurements on one cell at a time, it can process thousands of cells in a few seconds, giving a more representative idea of nature, and allowing more meaningful extrapolation. Since different cell types can be distinguished by quantitating structural features, flow cytometry can be used to count cells of different types in a mixture. Flow cytometry has great potential as a rapid, automated tool for ecological studies of micro-organisms. 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.

However, within the frame of MELGEN, it has been shown that it was impossible to distinguish a contaminant from a *R. rubrum* culture (TN70.11). A fluorescent probe was developped targetting against *R. rubrum*. By visualising *R. rubrum* by immunolabelled rRNA targetted oligonucleotide probes. It was expected to enable the separation between target and contaminating species on the basis of discriminating between fluorescent and non-fluorescent cells. However, the spirillum shaped bacterium intercallated with itself as well as with the contaminant. The cultures where hence difficult to resuspend, resulting in poor separation of the target and contaminating organism. On the other hand, flow cytometry presented to be an excellent tool to detect contaminating single cell organisms in the background of the filamentuous cyanobacterium *Arthrospira* sp..

Another way of detecting the presence of contaminants is to monitor changes in the metabolic parameters of the culture. Detection of respiration, metabolism, enzymes ... of the contaminants in the supernatant (for example, metabolisation of a specific substrate) might be used to find particular bacteria, using an indicator that changes of colour or fluoresces when cleaved from the specific substrate. This is used for coliforms (Hobson et al., 1996).

Detection of contaminants by proteomics: The proteomics techniques (Reverse phase capillary high performance liquid chromatography – electrospray ionisation mass spectrometry, gas chromatography-tandem mass spectrometry, MALDI-TOFS) are offering new developments to detect microbial contamination. Mass spectrometry in combination with novel bio-informatics provides a powerful new strategy for the rapid speciation and typing of microorganisms. This revolutionary Bacterial "Mass-Fingerprinting" approach offers greater sensitivity, selectivity and speed of analysis compared to classical identification methods in clinical microbiology, food science, biotechnology, water quality and pharmaceutical analysis. The method applies proven biopolymer Mass Spectrometry techniques to the analysis of intact bacteria the intact cell MALDI-TOF-MS (ICM-MS). The method allows the unique population of macromolecules expressed on the surface of bacteria to be rapidly sampled and characterised by molecular weight. The resulting mass spectrum provides a unique physico-chemical fingerprint for the species tested.

Mass-Fingerprints of unknowns 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 micro organism. Sample preparation is quick and easy - intact cells from primary culture are smeared across a stainless steel target plate and allowed to co-crystallize 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 micro organisms are desorbed and ionised. The resulting ionised macromolecules are mass analysed and the results reported as a mass spectrum - 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. This technique could be used to identify the bacterial population in Melissa compartment 1 and to characterize a possible contamination present in the other 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). Proteomic approach has been be used to identify the possible contamination from supernatant of bacterial cultures. 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 dilute samples before analysis by MALDI-TOF-MS, lectin-derivatized surface was 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 detecting trace levels of contaminating organisms.

Molecular methods are versatile tools and can be used (combined or not with visualisation by microscopy or flow cytometry) to detect the nucleic acids of the contaminants. Molecular techniques are already used in clinical medicine and can be adapted for environmental testing. Gene probes are being used that are highly specific and capable of detecting genetic sequences of DNA and RNA common to or conserved in pathogens such as *Salmonella* and *Legionella*, two disease-causing microbes. Researchers have now developed PCR, gene probes, and DNA "fingerprinting" techniques to detect intestinal bacteria and viruses in seawater and seafood" (Rose & Grimes, 2001). In general, PCR amplification must be thoroughly validated, as false-positive and false-negative results could occur (Vaneechoutte & Van Eldere, 1997). It is used to detect rapidly specific pathogens in samples, including viruses, slowly growing bacteria, fastidious or not-yet-cultivable bacteria, fungi and protozoa (Pillai, 1997). It is more difficult to use when the identity of the contaminants is unknown.

Most molecular tools used in clinical settings are targeting well known pathogens and enable a fast detection. Examples are the detection by amplification of 16S rDNA of *Bacillus cereus* group bacteria (Hansen *et al.,* 2001), *Staphylococcus aureus*, *Pseudomonas aeruginosa* and Enterobacteriaceae (Merker *et al.,* 2000), *Campylobacter* species (Magistrado *et al.,* 2001), *Staphylococcus aureus*, *Pseudomonas aeruginosa, Escherichia coli, Aspergillus niger* (Jimenez *et al.,* 2000).

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 –

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

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 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 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.

When PCR of species-specific genes is possible, a modification using Direct Labeling and Detection Procedure (DLDP) was shown by Gorelov *et al.* (1996) to detect less than 20 CFU of bacteria in human fluids. Other methods and kits have been published in the last years, targeting various bacteria in different kind of samples (food, clinical samples, etc). A DNA probe kit, using colorimetric DNA/rRNA sandwich hybridisation in microtiter wells was shown to detect 10^5 CFU/ml in pure culture of *Salmonella* spp (Namimatsu *et al.,* 2000). A chemiluminescent in situ hybridisation (CISH) using Peptide Nucleic Acids (PNA) probes could provide a fast detection of individual *Pseudomonas aeruginosa* or other bacterial microcolonies on membrane filters (Stender *et al.,* 2000; Perry-O'Keefe *et al.,* 2001). PNA molecules are pseudopeptides where the sugar phosphate backbone of DNA was replaced by a polyamide backbone. They behave like DNA for hybridisations but are more specific and stable. With the advent of Rapid-Cycle Real-Time PCR, PCR assays for routine use in clinical diagnostic testing are currently assayed and perform better in speed and sensitivity for most micro organisms tested (streptococci, *Bordetella pertussis*, diverse viruses) (Cockerill & Smith, 2002).

Although methods are increasingly sensitive and fast, there is one major drawback. Rarely studies have been performed on the detection of contaminating species in a background of a large volume of target organisms.

When the flow of recycling is followed in the MELiSSA loop, we can see that CII is at the most crucial position where contamination can occur. Therefore we should be able to detect any contaminant in a *R. rubrum* culture as soon as possible. In the nitrifying reactor, the most probable contaminant will be *R. rubrum*, due to leakage from CII to CIII. External contaminants are likewise possible, but less likely to occur, because the nitrifying reactor will not be opened during the entire operation. Henceforth, we should be able to detect *R. rubrum* in a background of nitrifying bacteria. In the third compartment, again any contamination should be spotted as early as possible in the algae compartment. Because the reactor will be opened for harvesting, also outside bacteria could enter the compartment, next to the possibility of contamination through leaking from CIII towards CIV.

In phase 1, *R. metallidurans* was used as a model bacterium to play the role of contaminant. Also because CIII was inaccessible, *R. metallidurans* played the role of the nitrifying bacteria, for the evaluation of contaminant detection methods.

The following spiking experiments were tested: (1) Contamination of CI: The contamination of the *R. rubrum* reactor by any contaminant. The *R. rubrum* suspension was contaminated with model contaminant *R. metallidurans*; (2) Contamination of CII: The contamination of the nitrifying consortium with *R. rubrum.* The model 'nitrifying bacteruim' *R. metallidurans* was contaminated with *R. rubrum*; (3) Contamination of CIII: The contamination of the algea *Arthrospira* sp. compartment with model contaminant *R. metallidurans.* The *Arthrospira* sp. suspension was contaminated with model contaminant *R. metallidurans*.

In MELGEN, 3 ways of detecting unknown contaminant microorganisms in a background of target species were evaluated: flow cytometry, intact cell mass spectrometry and PCR.

Detection limits using the three different methods are presented on the following table:

Detection method	**Detection limit (dilution ratios)**		
	R. met in *R. rub*	*R. rub* in *R. met*	*R. met* in *Arthro*
PCR	1/64	1/100,000	0
Flow Cytometry	1/256	1/64	1/1,000,000
MALDI-TOF analysis	1/64	1/256	1/1
		Arthro in *R. rub*	1/100

It was shown that while PCR was the most sensitive detection method when detecting *R. rubrum* cells in a *R. metallidurans* culture, flow cytometry was most sensitive in detecting *R. metallidurans* in an *Arthrospira* culture. MALDI-TOF analysis had not given any optimistic results. The method will not be improvable to detect the presence of contaminants. Flow cytometry was very sensitive to detect contaminants in the *Arthrospira* sp. compartment. This result, together with the ease of the manipulation of the samples can encourage the investigation on the detection of contaminants in *R. rubrum* with a strategy optimised for discriminating between the target organism and the contaminant. The pigmented *Arthrospira* sp. has several characteristics which can be of advantage to be used to discriminate between the microalgae and a possible contaminant: size, granularity and autofluorescence. The difference of the size and granularity between *R. rubrum* and a contaminant was not very clear. An improvement of the discriminating efficiency might be obtainable if we could label the target organism using a fluorescent marker targeted against DNA or a specific surface marker (either by using fluorescent probes or fluorescent antibodies). Identification of the surface proteins from *Ralstonia* and *Rubrum* has been conducted with the modified experimental set-up during the elution phase of the purification step in phase 1 of MELGEN. Within the project, GroEL and Dpsa like proteins have been identified as to surface proteins in *Rubrum* and *Ralstonia* respectively.

Recent work on *E. Coli* and *Helicobacter pylori* show also that GroEL can be surface associated protein. Indeed, polyclonal, monospecific antiserum was obtained for GroEL, which established by immunoelectron microscopy, indirect immunofluorescence and immunoblot analysis that GroEL is released extracellularly after heat shock and can be surface associated. Cell fractionation experiments suggest that GroEL is predominantly cytoplasmic and membrane bound. Two-dimensional electrophoretic and immunoblot analysis of cell surface proteins of spiral-shaped and coccoid forms of *Helicobacter pylori* indicate the presence of GroEL on the bacterial surface. These reports are in agreement with our observations. In conclusion, an antibody against GroEL of *rubrum* could be used to detect specifically this bacterium by flow cytometry. However, the specificity of the GroEL *R. rubrum* antibodies has to be tested. Indeed, a possible cross-reaction to *Arthrospira* GroEL is possible due to a high degree of a sequence similarity. In the future, other surface proteins will be identified by ESI-MS-MS. In this context, the biotinylated proteins will be separated by just a two-dimensional gel electrophoresis without a purification step as described in the literature. In the case of *Arthrospira* no result of biotynilation has been observed. The problems results of a bad lysis of this bacterium in the tested experimental conditions. The effectiveness of mechanical devices used to lyse a bacterium is clearly a function of the bacterial structure. The result of our observations is that in the case of manipulations of *Arthrospira*, the sonication step is not sufficient to obtain a complete lysis of this bacterium. MELGEN1 showed that a glass beads lysis (15 min. at room temperature) was far more efficient. In this context, this specific step allows a quantitative lysis of *Arthrospira*. In the future, this procedure will be used to identify surface proteins for *Arthrospira*.

The PCR based technique gave good results, revealing 1 *R. metallidurans* cell in a background of 100,000 *R. rubrum* cells. However, this technique seems only applicable when the contaminating organism is known. If this technique could be used to detect an unknown

contaminant, it would be very powerfull. Therefore the use of PNA probes, targeted specifically against the primary organism, is proposed to act as an inhibitor of PCR amplification, thereby allowing a positive discrimination on the amplification of contaminating DNA.

3 FUTURE DIRECTIONS

3.1 Fluorescent probes

3.1.1 INTRODUCTION

Fluorescence *in situ* hybridisation (FISH) on bacterial cells, using 16S rRNA targeted oligonucleotide probes is a very strong technique, which is particularly usefull for the evaluation of the phylogenetic identity, the morphology, the amount and the spatial distribution of the organisms in their environment (Amann *et al.,* 1995). In our experiments we would like to use a fluorescent probe specifically targeted against *R. rubrum*. Visualisation of the target-organism by increasing the fluorescence could subsequently improve the discrimination between target-organism and contaminant.

3.1.2 USING FLUORESCENT PROBES

The technique of fluorescence *in situ* hybridisation (FISH) targeted against 16S rRNA of bacterial cells allows simultaneous evaluation of the identity, the morphology, the number and even spatial arrangement of the microorganism. Probes can be designed to specifically target narrow to broad phylogenetic groups (from species to domain) based on the conserved and variable parts within the 16S rRNA molecule.

The FISH method involves the following steps: permeabilization of the cell, entering of the probe in the permeabilized cell and specific hybridisation to the complementary target sequence in the ribosomes. If no complementary target is found, the probe will be unable to hybridize, and unbound probe will be removed by a wash step. Therefore, only specifically targeted cells retain the probes under specifically stringent conditions. Labeled probes will hence visualise target organisms in a background of non target organisms.

3.1.3 THE EXPERIMENTAL STRATEGY

The aim in this chapter is to evaluate axenicity of the bacteria present in compartment II, *Rhodospirillum rubrum*, i.e. to detect the presence of contaminants, isolate them and characterise them (see Fig. 1).

The presence of contaminants is first detected by flow cytometry and fluorescent *in situ* hybridisation techniques. A fluorescent probe specific of *Rhodospirillum rubrum* is designed and *in situ* hybridisation is optimised to allow to differentiate *R. rubrum* from others possible microorganisms presents in the solution. This step will likewise be dependend on the optimisation of the detection of contaminants using flow cytometry.

Once the technique is optimised, The isolation of contaminants is done by a cell sorter, a tool similar to the flow cytometer. This equipment allows to detect, to separate and to collect the contaminants.

Finally, these contaminants are identified with the PCR (Polymerase Chain Reaction) based technique of TRFLP (Terminal Restriction Fragment Length Polymorphism).

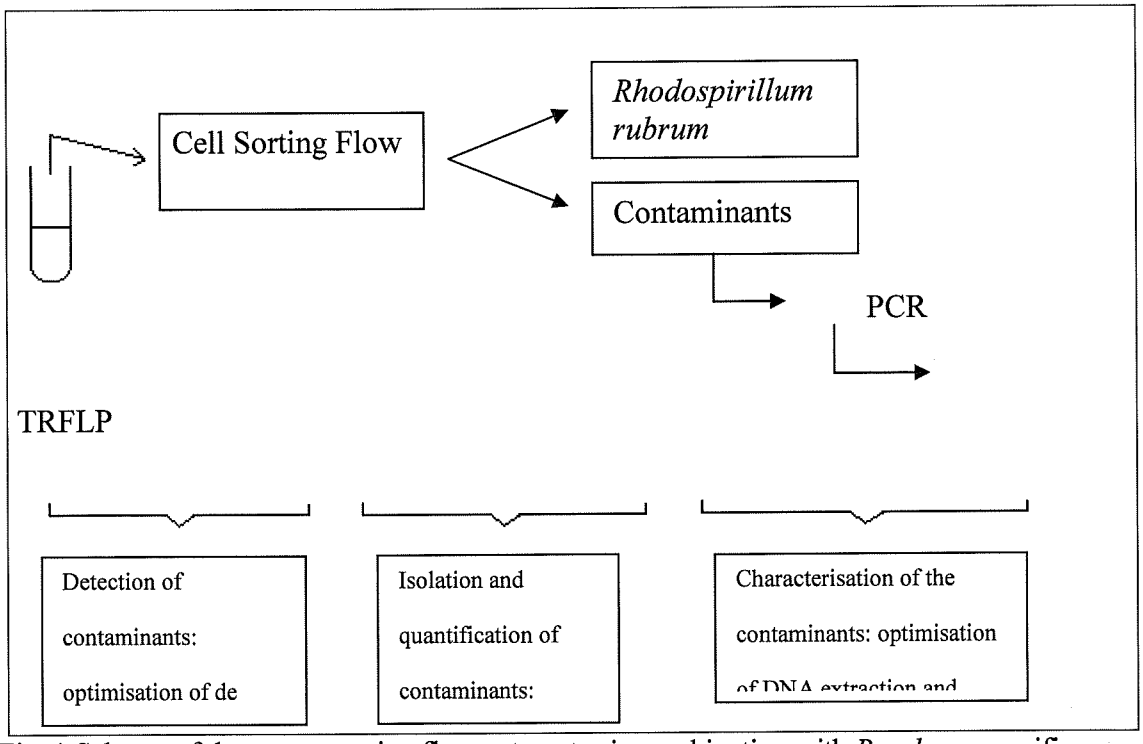


Fig. 1 Scheme of the strategy using flow cytometry in combination with *R. rubrum* specific fluorescent probes for the discrimination, isolation and characterisation of contaminants in a *R. rubrum* culture.

3.2 Fluorescent antibodies

3.2.1 INTRODUCTION

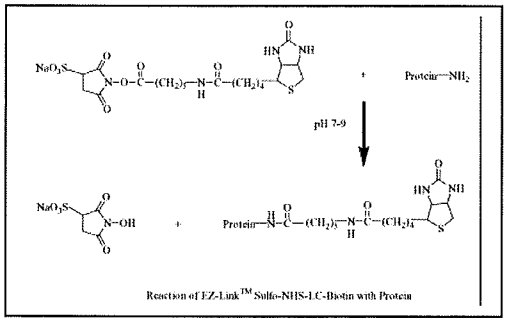
The major advantage offered by flow cytometry is that a specific cell population can be identified by staining with antibodies that are conjugated with fluorescent dyes (fluorochromes) and recognize membrane proteins (antigens). Cells can be differentiated depending on the expression of a protein marker especially a membrane specific protein. Using an antibody against specific bacterial surface proteins would improve the detection of contaminants in a specific culture, for instance in a MELiSSA compartment.

3.2.2 USING FLUORESCENT ANTIBODIES

The method of fluorescent antibody (FA) or immunofluorescence technique (IF) is a method using fluorescent markers appropriately conjugated to antibody proteins as they participate in antigen- antibody reactions. The method consists of the following steps: (1) a strain is used as an antigen for the preparation of active antiserum, (2) antiserum to the isolate is labeled with a fluorescent dye, and (3) the labeled antiserum is then applied as a stain to a sample containing the strain. If the microorganism of interest is present in the sample, the FA will combine specifically with it in an antigen-antibody reaction (Bohlool and Schmidt, 2000).

3.2.3 THE EXPERIMENTAL STRATEGY

The proteomic approach is one of the best approaches to identify a specific surface protein for each MELiSSA bacterial culture. A proteomic approach will be thus used to identify specific surface proteins for *R. rubrum*. During the MELGEN 1 project, the surface protein label protocol has already been adapted with success for *Ralstonia metallidurans* and *R. rubrum*.



The strategy to identify the surface protein involved 6 steps : (1) Specific label of surface protein by biotinylation, (2) Membrane protein extraction, (3) Purification by affinity chromatography, (4) Protein concentration by centrifugation, (5) Protein separation by electrophoresis, (6) Identification of protein by MALDI-TOF

Identification of the surface proteins from *Rubrum* has already been conducted during the melgen 1 project. Just now, GroEL protein has only been identified as to surface proteins in *R. rubrum.* The other membranes proteins will be identified during this project using the same strategy.

In function of the specificity of the membrane proteins, rabbit antibodies against one specific membrane protein will be realised. Finally, a coupling between the antibody and a fluorescence dye will be realised with a commercial kit.

3.3 PNA probes

3.3.1 INTRODUCTION

Over the last few years, the use of Peptide Nucleic Acids (PNA) as synthetic oligomers has proven their powerful usefulness in molecular biology. PNAs can be used in the same applications than traditional synthetic DNA or RNA, but with the added benefits of tighter binding and greater specificity. F. e., PNAs labeled with biotin, dioxigenin, fluorescent dyes or reporter enzymes are powerful probes in hybridization experiments (DNA array, Northern or Southern blot, FISH, detection of single point mutations, DNA mapping).

3.3.2 USING PNA PROBES

In our experiments we would like to use PNA as an inhibitor of specific 16S RNA gene PCR. Thereby enhancing the chance of a contaminant 16S RNA gene to be amplified more, in comparison with the *R. rubrum* 16S RNA gene.

This will be possible due to the characteristic chemical structure of the PNA oligo, providing it with increased stability due to stronger binding. The backbone is made from repeating N-(2-aminoethyl)-glycine units linked by peptide bonds. The different bases (purines and pyrimidines) are linked to the backbone by methylene carbonyl linkages. Unlike DNA or other DNA analogs, PNAs do not contain any pentose sugar moieties or phosphate groups.

3.3.3 THE EXPERIMENTAL STRATEGY

The first step in this strategy is to design a probe specific for *R. rubrum*. Thereafter a PCR protocol should be optimised to use the PNA probe in a PCR experiment. In a last step the method will be tested on DNA extracted from a semi-sterile running *R. rubrum* reactor.

(A) Design of the probe
- ARB design
- *In silico* analysis

(B) PCR optimisation
- temperature
- time cycles
- conc of PNA
- conc of DNA
- primerpair combinations

(C) Application of the method
- PCR with PNA
- Subsequent DGGE analysis

3.4 Precise estimation of the limits of detection of axenicity by flow cytometry in MELGEN bacterial strains

3.4.1 COMPARISON OF BACTERIAL COUNTING BY FLOW CYTOMETRY, PLATING AND OPTICAL DENSITY ON MELGEN BACTERIAL STRAINS

Spiking experiments have been performed during MELGEN 1 in order to compare various techniques (PCR, MALDITOF and flow cytometry) and define their thresholds of sensitiviy. We could conclude from those experiments that flow cytometry was able to detect contaminants up to a certain threshold which was in certain conditions even lower than the two other techniques cited above. However, one question that remains is the precise numbers of cells of MELGEN bacterial strains that were used in order to perform the spiking tests. Spiking experiments were performed taking into account the optical density (OD) of the bacterial cultures. The OD does not always give a precise estimation of the number of cells used for the experiments. Indeed, it is known that the OD varies in function of the size of the organism, the composition of the medium (presence of cristals and protein aggregates). Therefore, we think that it would be of interest to develop a method by flow cytometry which will be compared with plating and optical density of MELGEN bacterial strains in order to precisely estimate the number of contaminants. Flow cytometry counting (with two different methods available on the market) will be compared to the optical density and the plating. The three techniques will be applied in standard culture conditions on *R. metallidurans*, *E. coli* and *R. rubrum.*

3.4.2 COMPARISON OF CELL SIZE ESTIMATION BY MICROSCOPY AND FLOW CYTOMETRY

The forward scatter used in flow cytometry is proportional to the cell size of the organism. However, when bacteria are passing through the flow cytometry noozzle, they are more or less aligned in the flow stream meaning that the instrument measures the width of the organism. However, we would like to compare whether the values obtained by the flow cytometer reflects the real biological sizes of the MELGEN bacterial strains. For that purpose, it is planned to compare microscopy values with flow cytometry on the MELGEN strains.

3.5 References

Amann R. I., Ludwig W., Schleifer K. H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev. 59(1):143-69.

Bright JJ, Claydon MA, Soufian M, Gordon DB. (2002). Rapid typing of bacteria using matrix-assisted laser desorption ionisation time-of-flight mass spectrometry and pattern recognition software. J Microbiol Methods. 2002;48(2-3):127-38.

Bundy J, Fenselau C. (1999). Lectin-based affinity capture for MALDI-MS analysis of bacteria. Anal Chem. 71:1460-3.

Cockerill, FR, Smith, TF (2002). Rapid-cycle real-time PCR: a revolution for clinical microbiology. ASM News, 68:77-83.

Fenselau C, Demirev PA. (2001). Characterization of intact microorganisms by MALDI mass spectrometry. Mass Spectrom Rev. 20:157-171.

Gorelov VN, Dumon K, Barteneva NS, Roher HD, Goretzki PE. (1996). A modified PCR-based method for rapid non-radioactive detection of clinically important pathogens. Microbiol Immunol. 40:611-6.

Gunasekera TS, Attfield PV, Veal DA. (2000). A flow cytometry method for rapid detection and enumeration of total bacteria in milk. Appl Environ Microbiol. 66:1228-32.

Hansen BM, Leser TD, Hendriksen NB. (2001). Polymerase chain reaction assay for the detection of *Bacillus cereus* group cells. FEMS Microbiol Lett. 2001, 202(2):209-13.

Hobson NS, Tothill I, Turner AP. (1996). Microbial detection. Biosens Bioelectron. 11:455-77

Jimenez L, Smalls S, Ignar R. (2000). Use of PCR analysis for detecting low levels of bacteria and mold contamination in pharmaceutical samples. J Microbiol Methods. 41:259-65

Jimenez L. (2001). Molecular diagnosis of microbial contamination in cosmetic and pharmaceutical products: a review. J AOAC Int. 84:671-5.

Johnson PE, Lund ML, Shorthill RW, Swanson JE, Kellogg JL. (2001). Real time bio-detection of individual pathogenic microorganisms in food and water.Biomed Sci Instrum. 37:191-6.

Kawano Y, Ito Y, Yamakawa Y, Yamashino T, Horii T, Hasegawa T, Ohta M. (2000). Rapid isolation and identification of staphylococcal exoproteins by reverse phase capillary high performance liquid chromatography-electrospray ionization mass spectrometry. FEMS Microbiol Lett. 189:103-8.

Laplace-Builhe C, Hahne K, Hunger W, Tirilly Y, Drocourt JL. (1993). Application of flow cytometry to rapid microbial analysis in food and drinks industries. Biol Cell. 78:123-8.

Lay JO Jr. (2000). MALDI-TOF mass spectrometry of bacteria. Mass Spectrom Rev. 20:172-94.

Magistrado PA, Garcia MM, Raymundo AK. (2000). Isolation and polymerase chain reaction-based detection of *Campylobacter jejuni* and *Campylobacter coli* from poultry in the Philippines. Int J Food Microbiol. 70:197-206

Merker P, Grohmann L, Petersen R, Ladewig J, Gerbling KP, Lauter FR.(2000) Alternative microbial testing: a novel DNA-based detection system for specified microorganisms in pharmaceutical preparations PDA J Pharm Sci Technol.;54(6):470-7.

Namimatsu T, Tsuna M, Imai Y, Futo S, Mitsuse S, Sakano T, Sato S. (2000). Detection of *Salmonella* by using the colorimetric DNA/rRNA sandwich hybridization in microtiter wells.
J Vet Med Sci. 62:615-9.

Perry-O'Keefe H, Stender H, Broomer A, Oliveira K, Coull J, Hyldig-Nielsen JJ.(2001). Filter-based PNA in situ hybridization for rapid detection, identification and enumeration of specific micro-organisms. J Appl Microbiol. 90(2):180-9.

Pillai SD. (1997). Rapid molecular detection of microbial pathogens: breakthroughs and challenges. Arch Virol Suppl. 13:67-82.
Rose, J.B. & Grimes, D.J. Reevaluation of microbial water quality : powerful new tools for detection and risk assessment. American Academy of Microbiology (2001).

Saxena J, Munimbazi C, Bullerman LB. (2001). Relationship of mould count, ergosterol and ochratoxin A production. Int J Food Microbiol. 71:29-34

Stender H, Broomer A, Oliveira K, Perry-O'Keefe H, Hyldig-Nielsen JJ, Sage A, Young B, Coull J. (2000). Rapid detection, identification, and enumeration of *Pseudomonas aeruginosa* in bottled water using peptide nucleic acid probes. J Microbiol Methods. 42:245-53
Szponar B, Larsson L. (2001). Use of mass spectrometry for characterising microbial communities in bioaerosols. Ann Agric Environ Med. 8:111-7.

Takeuchi K, Frank JF. (2001). Confocal microscopy and microbial viability detection for food research. J Food Prot. 64:2088-102.

Vaneechoutte M, Van Eldere J. (1997). The possibilities and limitations of nucleic acid amplification technology in diagnostic microbiology. J Med Microbiol. 46:188-94.