MELiSSA

TECHNICAL NOTE

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PART B: TEST REPORTS FOR AXENICITY STUDY, RUN AS ANNOTATED TEST PROCEDURES

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ABREVIATIONS

BHR	Broad-Host Range
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
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
NCTC	National Collection of Type Cultures (Colindale, UK)
OD	Optical Density
PCR	Polymerase Chain Reaction
PMT	photomultiplier
rDNA	rRNA encoding DNA
RPM	Revelations Per Minute
RMS	root mean square
SAPD	Surface Accessible Proteins Detection
SCM-MS	Supernatant MALDI-TOF MS
SS	side scatter
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TCA	TriChloroacetic Acid
TLF	Time Lag Focusing
Tris	Tris(hydroxymethyl)-aminomethane
UV	Ultra Violet

1 INTRODUCTION

It is important to control the axenicity of the MELiSSA compartments because contaminants constitute a major concern in the proper functioning and maintenance of a closed artificial ecosystem. Contamination by biomolecules, normally not produced in a given compartment, may result into instabilities or even the collapse of the system, rendering the biomasses of *Arthrospira* sp. unsuitable for human consumption. In addition, contamination of C4 by pathogenic organisms almost certainly will pose serious risks to human health. One should also keep in mind that the liquid medium is recycled, causing an enrichment in organics and metabolites. This may favour the development of contaminants or may give rise to unstable reactor conditions.

Biological contaminants can be of bacterial, fungal, protistic, prion or viral origin. Metazoan contamination (e.g. molluscs, nematodes, rotifera, etc.) of the MELISSA cycle is rather unlikely. However, the risks mediated by emerging pathogens and other infectious agents (e.g. prions) should be assessed.

In this technical note the optimisation of the methods are described to detect the presence of contaminants in *R. rubrum* ATCC25903 and *Arthrospira* sp. PCC8005.

2 OPTIMISING FLOW CYTOMETRY FOR DETECTION OF BACTERIA

2.1 Introduction

In this chapter are presented the different optimisation protocols required to guarantee an optimal and reliable measurement of bacteria by flow cytometry. The first part is devoted to the description of the optimization of the alignment, the focusing and the calibration of the flow cytometer device whilst the second part concerns the enumeration, detection of bacterial size and shape (granularity) and the explanation of the protocol followed for the spike experiments.

2.2 Flow cytometry: technical optimisation

2.2.1 OPTIMISATION OF THE FLOW RATE OF SAMPLE CELL INSIDE THE FLOW CYTOMETER

As already mentionned in the TN 70.80, 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. Therefore, it is very important to adjust the speed in order to obtain the best discrimination. When the rate is too high, the sharpness of the peaks decreases. When the rate is too low, the experimental time is too long. During this experiment, an optimal cell flow rate needed to be determined empirically. With the flow cytometer EPICS XL, we have currently the choice between three speeds : low, medium and high. All the samples were passed within the flow cytometer with a low speed that gave the best discrimination.

2.2.2 FLOW CYTOMETRY ALIGNMENT VERIFICATION

The flow cytometer is designed to perform quantitative measurements on individual cells and other particles with high precision, speed and accuracy. As with all high-performance instrumentation, our flow cytometer had to be calibrated at least daily to verify the flow cytometer's optical aligment and fluidics system which ensures accuracy and reliability. For this purpose, we use every day the Flow-Check fluorospheres which consists of an assayed suspension of fluorospheres (fluorescent microspheres) with uniform and stable size and fluorescence intensity and suspended in an aqueous media containing surfactants and preservatives at 1 x 10^6 fluorospheres/mL (nominal concentration). The fluorescence emission of the dye contained within the fluorospheres ranges from 525 nm to 700 nm when excited at 488 nm. The stability, uniformity and reproducibility of these fluorescent fluorospheres make them ideal daily reference standards. Furthermore, the use of those beads permits the calibration of our flow cytometer's laser, optics and stream flow without wasting our valuable experimental material. These fluorospheres are designed to approximately replicate the size, emission wavelength and intensity of biological samples. Because the dyes are contained

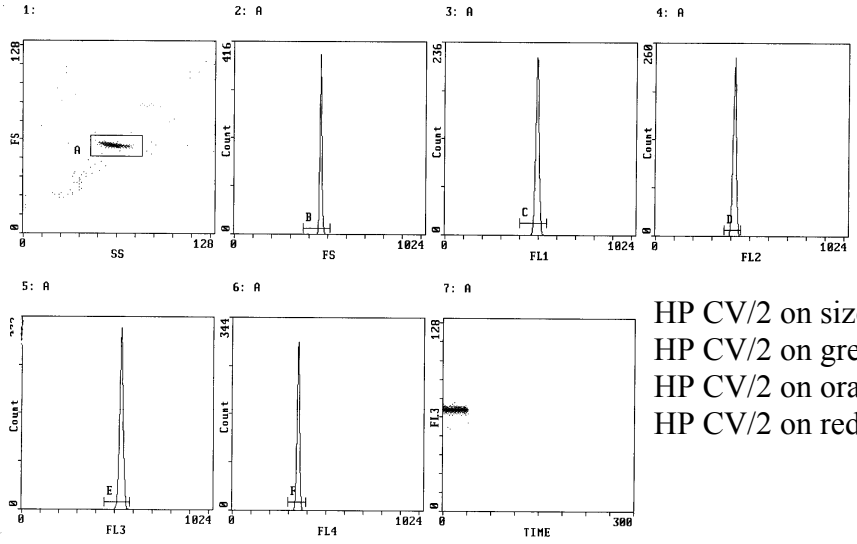
inside the microsphere's matrix, instead of on the bead's surface, Flow Check fluorospheres have excellent photochemical and physical stability, providing reliable reference signals for aligning, focusing and calibrating our flow cytometer.

Procedure for daily verification of alignment and fluidics.

1. Select the Flow-Check fluorospheres test protocol (QA QC – ZxyzFLOW.QCC).
2. Vigorously mix the Flow-Check fluorsopheres vial until no sediment is observed on the bottom of the vial.
3. Dispense 15-20 drops (about 0.5 mL) of Flow-Check fluorospheres into a test tube.
4. Vortex and aspirate the fluorospheres sample from the test tube.
5. If necessary, adjust the appropriate detector settings to place each peak within the peak position range established.
6. Record the half peak CV (HPCV) and peak position for each desired parameter.
7. Record the daily HPCV values for each desired parameter on its respective Levey-Jennings graph.
8. Ninety-five percent of values should fall within the ± 2 SD range for each parameter.

Expected results.

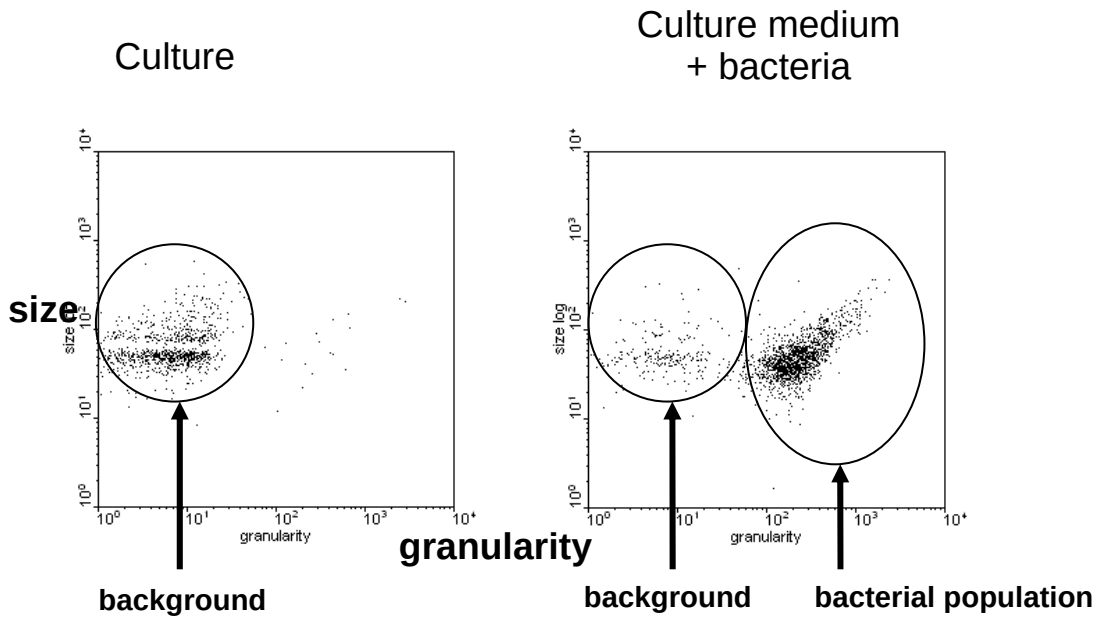
Generally, a HPCV of <3% is acceptable for surface marker applications, and <2% is acceptable for DNA applications. The figure below represents a daily measurement of the Flow-Check fluorospheres. All the HP CV are under 2%.



HP CV/2 on size : 0.92 %
HP CV/2 on green fluo : 1.59 %
HP CV/2 on orange fluo : 1.33 %
HP CV/2 on red fluo : 1.51 %

2.2.3 DISCRIMINATION OF THE BACTERIAL CELLS FROM THE BACKGROUND

When samples are not stained, the approach we developed was to start with a bacterial suspension. The acquisition was started and the needed photomultipliers (PMT 1 to PMT 4) were increased in order to obtain the main bacterial population in the middle of the screens. In all cases, it was critical to adjust the photomultiplier values in order to use all the dynamic range of the logarithmic scales. Then a flow cytometric tube containing sheath (Isoton^{TM}) was used. The discriminator was set on the size (forward scatter) at the minimum value. The discriminator was increased until most of the background could be avoided. The total number of events per second was maintained below 1000. Then the bacterial sample was run again and the discriminator adjusted again. The total number of events per second must be maintained below 1,000 per second. Since bacteria are very small, it is hard to completely avoid having background. This is however easily circumvented by the fact that it is possible to gate the bacterial populations that will be further analysed. Figure 2.2 below shows a flow cytometrical view of bacterial populations and how easy it is possible to distinguish it from the background emitted by the medium.



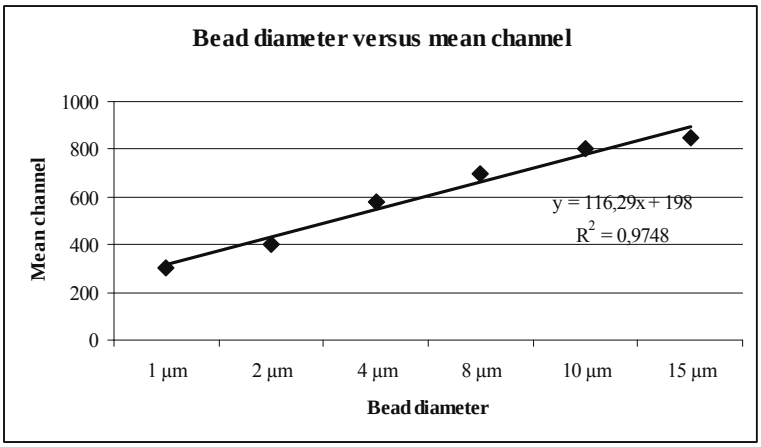
2.2.4 DATA ACQUISITION

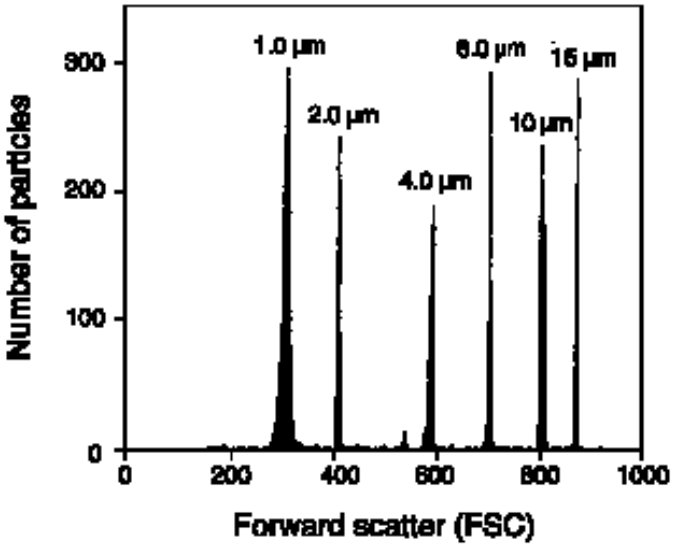
Samples were collected as listmode and routinely 100,000 to 200,000 events were recorded typically during 2 to 10 minutes on an EPICS XL flow cytometer using the low (or eventually the medium) sample flow rate.

2.3 Basic detection of differences in size and granularity of the MELISSA strains

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. In this study, the aim was to analyse whether or not a change in the bacterial culture (due to temperature, oxidative or pH stresses) could be correlated with a change in size and granularity.

As shown in figure 2.3. and figure 2.4., changes were quantified by the use of calibrated microspheres of known diameter size (1, 2, 4, 8, 10 and 15 µm diameter) and a calibration curve of the mean channel versus the size was established. If there is a change of the bacterial size, it should be reflected by a change in the FS (forward scatter = size) and SS (side scatter = granularity) means. For information, tests have not been performed with beads of diameter size < 1 µm, so far. However, it is intended to perform this test in the future.



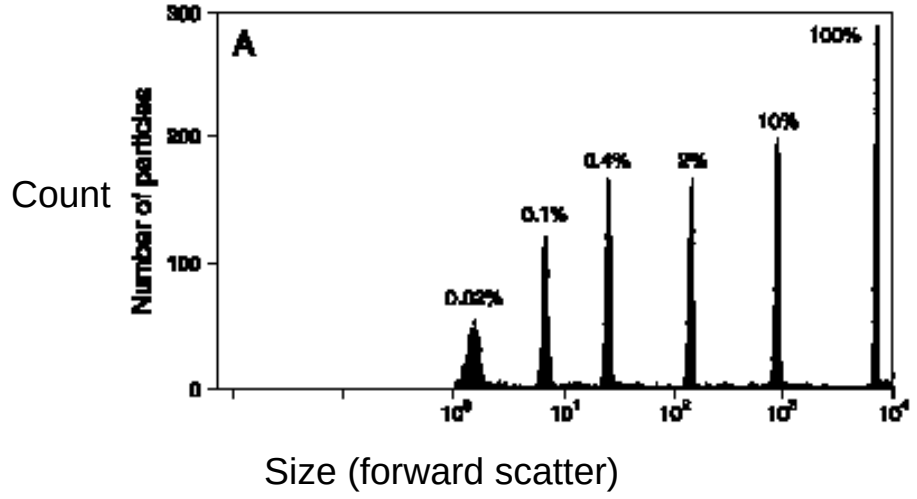


2.4 Basic detection of differences in green fluorescence of the MELISSA strains

For the calibration of green fluorescence, we used the LinearFlow™ Green Flow Cytometry Intensity Calibration Kit. This kit contains six suspensions of fluorescent polystyrene microspheres. Each of the six microsphere samples produces a different precisely determined intensity level when excited in our flow cytometer (using 488 nm emission). The microspheres have an emission maximum of ~515 nm, closely matching that of samples labeled with fluorescein and were useful for calibrating the green (FL1) channel of our flow cytometer. The microspheres actually have an excitation maximum of ~505 nm; however, the beads are effectively excited by the 488 nm spectral line of the argon-ion laser.

Each LinearFlow Green Kit includes six vials of fluorescently labeled polystyrene beads that have been stained with a fluorescent dye at different intensity levels.

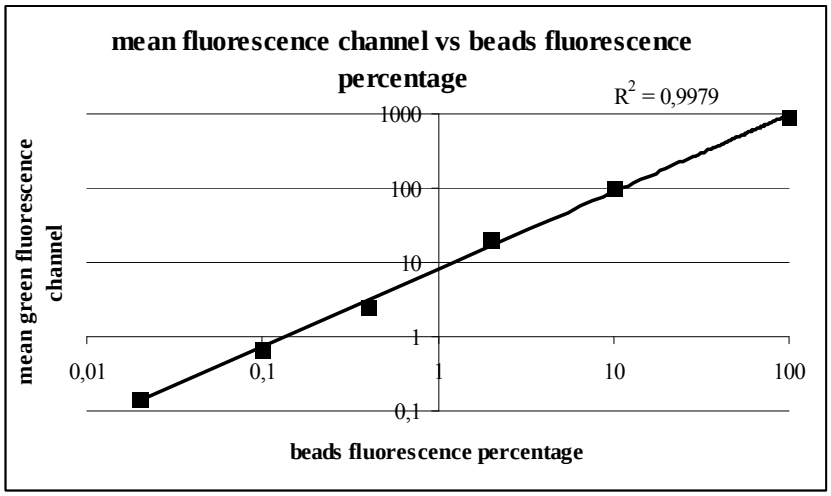
By using these standard microbeads, we performed quantitative quality control and measurements. The quantitative measurements include mean fluorescence intensity values (MFI in linear values or channel numbers) which can be transformed into calibrated values of Molecules of Equivalent Soluble Fluorochrome (MESF). The following fluorescence intensity histogram of the six polystyrene bead samples supplied in our LinearFlow Green Flow Cytometry Intensity Calibration Kit.



Experimental protocol.

1. Before sampling any of the kit components, vortex the contents to ensure that the beads are uniformly suspended.
2. One drop from each vial added to 2 mL of buffered saline solution provides an appropriate bead density for analysis.
3. Sonicate the diluted bead suspension to disperse aggregates and vortex immediately before use.
4. Using the LinearFlow beads, adjust the photomultiplier (PMT) settings, laser, optics and stream flow in accordance with the flow cytometer operating instructions.
5. The six intensity peaks corresponding to the kit components should be well separated and sharply defined. The relative position of the standard peaks can serve to evaluate the fluorescence of biological samples. Samples can either be intermixed with the reference beads or analyzed in subsequent runs in the flow cytometer.

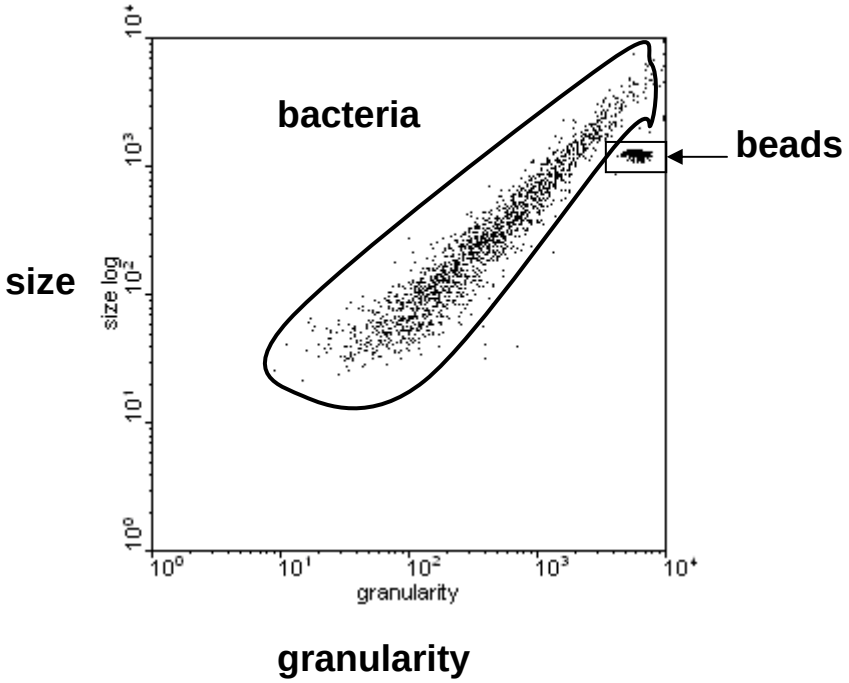
The following graph shows the mean fluorescence channel in function of the known bead fluorescence as they appear in our hands. The regression curve shows a good correlation from low to high fluorescences.



This calibration is important for all green fluorescences measurements and was used in our experiments to estimate e.g. DIOC6(3), Rhodamin-123, FDA, cFDA fluorescences. However, this methodology did not allow us to estimate pigment fluorescence (but this is mainly due to the limitation of fluorescence detection of the particular flow cytometer we used for these experiments).

2.5 Flow cytometric enumeration of bacteria.

Accurate enumeration of bacteria in a sample was an important aspect of some of our experimental procedures. The bacterial cell counting was performed with the help of the FlowCount kit which consists of a suspension of polystyrene microspheres that serve as a reference standard to indicate sample concentration. The calibrated suspension of beads in the microsphere standard has size characteristics and relatively low fluorescence appropriate for use in combination with any type of bacteria that may be present in our samples. The Flow Count Kit was particularly valuable for monitoring bacterial growth, as it provides a convenient and accurate means for assessing changes in a bacterial population over time. A definite volume of FlowCount reagent (containing a definite number of beads) is simply diluted in a definite volume of bacterial suspension. Bacteria and microsphere particles are easily distinguished in a plot of forward scatter versus fluorescence; the density of bacteria in the sample can be determined from the ratio of bacterial signals to microsphere signals in the dot-plot. The following figure shows the flow cytometric enumeration of *Ralstonia metallidurans* using the FlowCount kit (figure 2.6.). In this plot of forward scatter versus fluorescence, signals in the lower left hand frame represent *R. metallidurans* bacteria; signals in the upper right hand frame represent microsphere particles, which serve as a standard used to indicate sample concentration.



Sample preparation

1. Dilute the bacterial culture with filter-sterilized isoton buffer to a final density of about 10^6 cells/mL. A 1.0 mL volume will be used for each assay.

2. Thoroughly resuspend the microsphere standard suspension by sonication in a waterbath for about 5 minutes. Add 20 µL of the microsphere suspension to 1.0 mL of the bacterial suspension, mix well and analyze by flow cytometry.

3. Bacterial counting can be assayed in a flow cytometer. Fluorescence of the beads is collected in the four fluorescence channels. The forward scatter and fluorescences should be collected with logarithmic signal amplification.

4. Apply an experimental sample (bacteria plus microspheres) and process the data by framing two regions in the forward scatter versus side scatter dot-plots (see hereabove). Counting the number of signals in the microsphere frame provides an accurate estimate of the volume analyzed in the data file. The microsphere standard contains 1.0×10^6 beads/mL; thus, after ~50-fold dilution (20 µl into 1.0 mL) into the bacterial preparation, the microsphere density is 0.5×10^4 beads/mL. The number of signals in the bacteria frame divided by the number of signals in the microsphere frame provides the number of bacteria per 10^{-6} mL of the sample.

Sometimes, in the case of very dilute bacteria samples, long data-acquisition times were required. In those cases, the number of noise signals acquired in the bacteria frame could become significant. Therefore, a control sample (consisting of medium with beads but without bacteria) was run for the same time duration. The results attained with the control sample provided us an estimate of the noise contribution in the experimental data files.

For information, knowing the flow rate (5, 30 or 60 µl/min for the low, medium or high rates, respectively) as well as the start volume to boost the measurement (around 100 µl) can help determining the bacterial concentration of a sample. However, it is not considered by experts as being precise enough since it may depend on the volume of sheath present in the machine (if it is high, the following volume and concentration are correct but if the sheath level is too low in the machine, those values are not reliable anymore). Nevertheless, it is sometimes used as a rough measurement.

2.6 Optimisation for the spike experiments

Figure 2.7. shows the principle of the detection of the two populations of *R. rubrum* and *R. metallidurans* by flow cytometry used for the spike experiment. First, we passed the samples containing either *R. rubrum* or *R. metallidurans*. For each population, we drew a region corresponding to the population (here in red for *R. rubrum* and in blue for *R. metallidurans*). A certain region of the populations overlap. However, each populations has also a part which is specific and that is the region we take into account for the spiking experiment in order to determine the threshold of "contamination" of *R. rubrum* by *R. metallidurans* and vice versa.

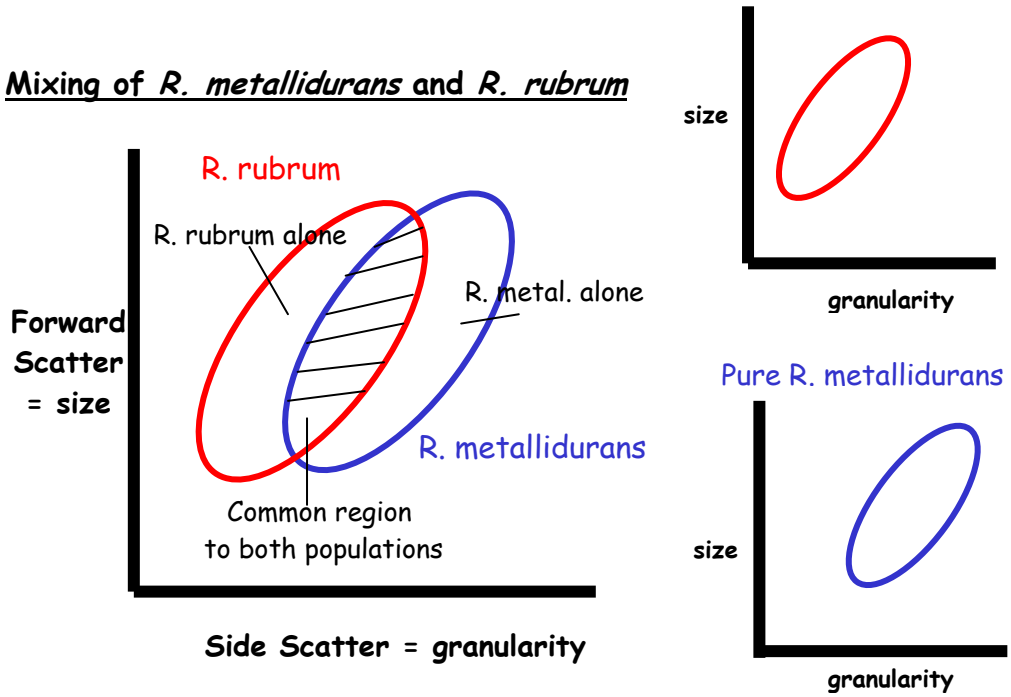
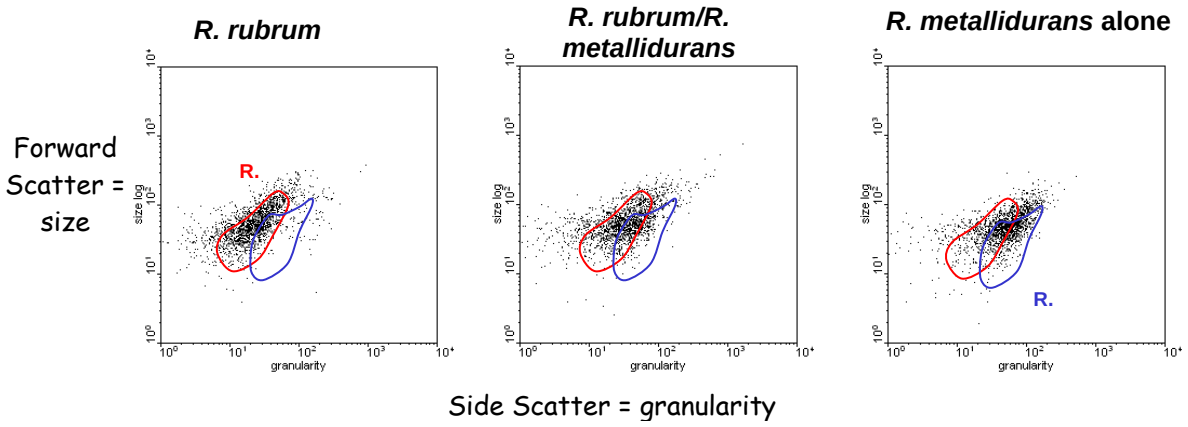


Figure 2.8. shows typical samples of *R. rubrum* and *R. metallidurans* alone or in a combined culture in a 1:1 ratio.



2.7 Conclusions

Flow cytometry was optimized to detect and count bacteria. Discrimination of bacteria from the background was performed according to their size and shape. Protocols were then set-up to count bacteria and to distinguish contaminants for the spike experiments (TN 70.11). In the future experiments, various protocols using fluorescent labeling will be developed in order enhance discrimination between bacterial strains.

3 CHARACTERISATION OF BACTERIA BY MASS SPECTROMETRY: VALIDATION OF THE MALDI-TOF

3.1 Introduction

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. This mass fingerprint is characteristic of the particular species, and in some cases differences at the strain level can be observed. 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" (Intact cell MALDI-TOF-MS (ICM-MS)) 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.

In this context, the ICM-MS approach will be used to detect surface modifications during stress conditions and to study the axeny in long term microbial cultures.

During this project, a new MALDI-TOF MS system has been acquired. After a training period, this system has been validated: first , for the protein identification and second, for the bacterial characterization by the ICM-MS approach. These validation have been realised on *Ralstonia metallidurans*.

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.

3.2 Validation of the MALDI-TOF on model organism Ralstonia metallidurans

The MALDI-TOF has been tested to characterize the bacteria from intact cells. This validation has been realized, in the first time, on ***Ralstonia metallidurans.***

3.2.1 SAMPLE PREPARATION

Different sample preparation conditions have been tested. First, *Ralstonia metalidurans* CH34 strain has been cultured on Mineral salts medium supplemented with 0.2% (w/v) gluconate, containing 2% (w/v) agarose. These plates were incubated for 54 h at 30°C. Second, bacterial strains were sub-cultured on liquid medium as in the MELISSA compartments for 54 h at 30°C and centrifuged just before the analysis. In both cases, after centrifugation (at 30°C, 5000 RPM during 5 min.), bacteria were resuspended in 200 ul of water (milli-Q) and were spotted on the MALDI plate (2 ul by sample). The samples were then dried at 37°C during 1h. After this step, 2 ul of MALDI-tof Matrix solution were added and dried at room temperature. For each matrix solution, 12 samples were analysed. The ionisation process depends of the nature of the matrix solution and the bacterial type. In this context we have tested 4 different matrix solutions. Twelve target wells were used for each organism to be entered into the database. In this study, 4 different matrix solutions were used :

A) alpha1 : saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) both dissolved in 1:1:1 water, acetonitrile and methanol with 0.1% (v/v) formic acid and 0.01 M 18-crown-6. The formic acid promotes positive ion production and 18-crown-6 removes metal ion adducts.

B) alpha2 : saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) both dissolved in 1:1:1 water, acetonitrile and methanol with 0.1% (v/v) formic acid

C) alpha2 : saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) both dissolved in 1:1:1 water, acetonitrile and ethanol with 0.1% (v/v) formic acid

D) alpha2 : saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) both dissolved in 1:1:1 water, acetonitrile and butanol with 0.1% (v/v) formic acid

3.2.2 MASS SPECTROMETRY MEASUREMENTS

Mass spectrometry measurements were performed on a M@LDI LR (Micromass, Manchester, UK) laser desorption time-of-flight instrument equipped with a nitrogen UV laser (λ=337 nm). The mass spectrometer was used in the positive ion detection mode using an acceleration voltage of +15kV and a linear mode. On loading each target plate, automatic, accurate indexing of sample/reference wells was performed, followed by calibration of the m/z range of the instrument, using the average molecular weights from a standard peptide/protein mixture (Bradykinin, angiotensin, Glu-fibrinopeptide B, renin substrate tetraddecapeptide, ACTH (18-39) all at 1 pmol/ul, bovine insulin, 2pmol/ul). The acquisition mass range was from m/z 500 to 10000 Da. For maximum throughtput of samples the bacterial mass fingerprints were acquired automatically.

3.2.3 DATA ANALYSIS

Spectral data were analysed with the MicrobeLynx™ algorithm and compared with a specific database. A bacterial fingerprinting database (>1500 different bacterial species) is accessible from the Manchester Metropolitan University in collaboration with the National Collection of Type Cultures (NCTC), Central Public Health (London). The quality of the replicates was determined by comparing each of the twelve replicate spectra to each other using the root mean square (RMS) value. An RMS rejection value of 3 used to identify significant outliers and an average mass spectral fingerprint obtained from a minimum of ten accepted replicate spectra of an individual bacterium was used to populate the database. The pattern recognition algorithm uses all the mass and intensity data in the mass spectrum to give the best database match with a probability score.

3.3 Results

3.3.1 REPRODUCIBILITY OF THE FINGERPRINT OF *RALSTONIA METALLIDURANS*

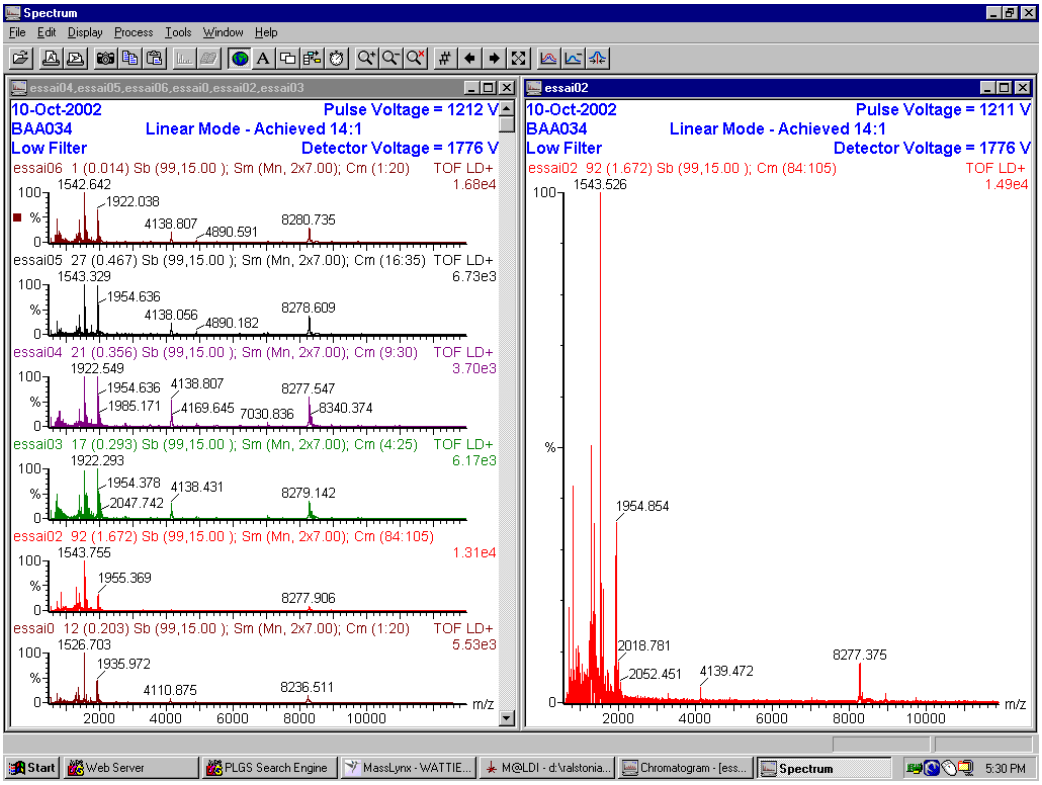


Fig. 3.1. MALDI-TOF fingerprint of intact *Ralstonia metallidurans*

The reproducibility of the intact bacteria fingerprint was realised from 6 different cultures on minimum medium (agarose minimum medium, 3 days of incubation at 30C) and directly spotted on the MALDI plate. In this test, the matrix alpha1 was used. This matrix was used to build the international bacterial database (Manchester Metropolitan University). In our experimental conditions, 99.9 % of reproducibility between the different fingerprints was obtained.

3.3.2 SPECIFICITY OF THE FINGERPRINT OF *RALSTONIA METALLIDURANS.*

The specificity of the fingerprint of *Ralstonia metallidurans* was tested by comparison with the bacterial fingerprint international database, after the automated fingerprint analysis with the "pattern recognition algorithm".

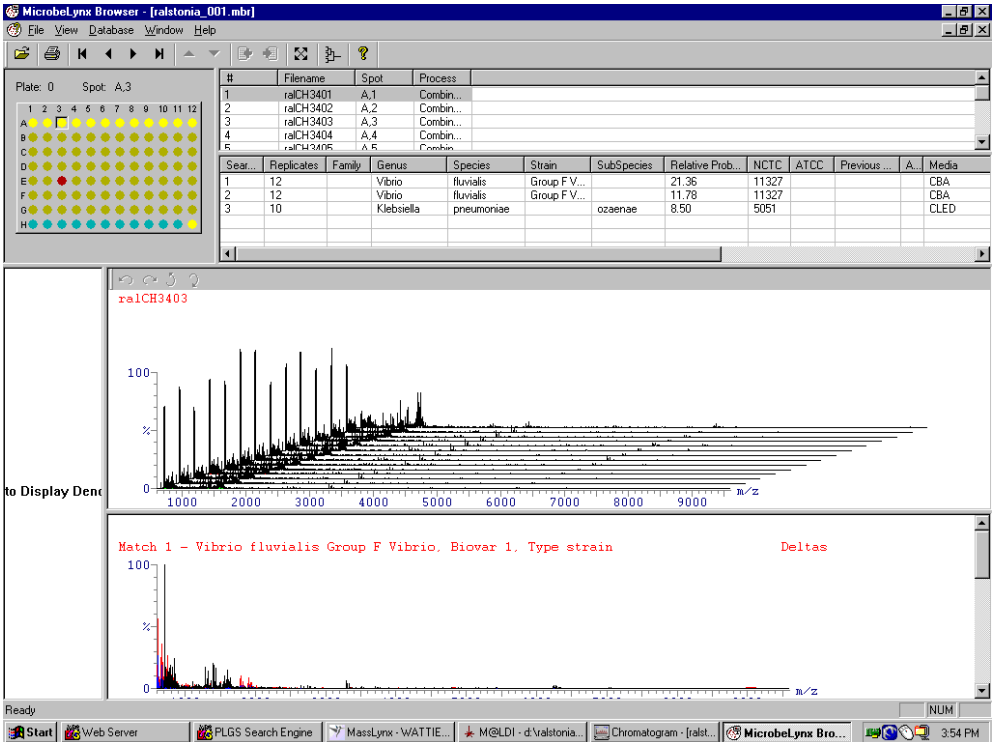


Fig. 3.2 Automatic comparison of *Ralstonia metallidurans* CH34 fingerprint against an international microbial database (>1500 different bacteria) with the Microbiolynx ™ system

The quality of the replicates was determined by comparing each of the twelve replicate spectra of *Ralstonia metallidurans* to each other using the root mean square (RMS) value. An RMS rejection value of 3 used to identify significant outliers and an average mass spectral fingerprint obtained from a minimum of ten accepted replicate spectra of an individual bacterium was used to populate the database. The pattern recognition algorithm uses all the mass and intensity data in the mass spectrum to give the best database match with a probability score. This system was completely automated. For *Ralstonia metallidurans*, any significant

matching was observed, indeed this bacteria was not present in the international fingerprint database.

In other part, a bacterial fingerprint database containing all fingerprints of 1500 different bacteria and the fingerprint of *Ralstonia metallidurans* CH34 was build. In this context, 96 different cultures (3ml) of *Ralstonia metallidurans* CH34 was analysed by the MALDI-TOF and a average mass spectral fingerprint obtained by the Microbiolynx algorithm was introduced in the database. To test the pattern recognition algorithm (microbelynx ^{TM}), samples of *Ralstonia metallidurans* CH34 were submitted to the intact cell MALDI-TOF analyse and compared against our new database. As shown the figures 3.2. and 3.3., a significant matching (100% probability) was observed with the fingerprint of *Ralstonia metallidurans* CH34. This experiment was duplicated with 6 different cultures of *Rasltonia* and a similar probability was observed.

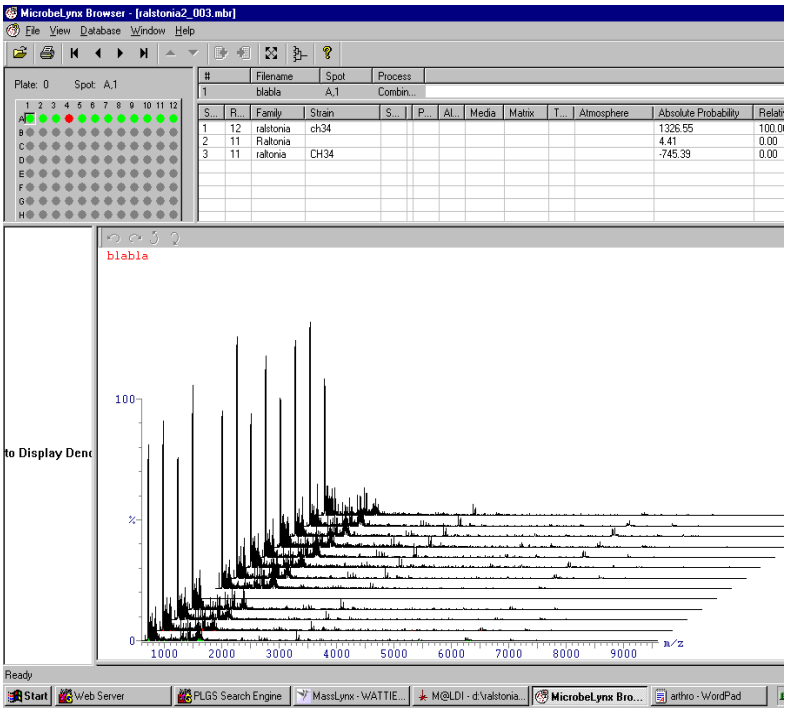


Fig. 3.3. Automatic comparison of *Ralstonia metallidurans* fingerprint against our new database with the Microbiolynx ^{TM} system.

3.4 Modifications of the intact bacterial fingerprint by environmental variations: Validation on Ralstonia metallidurans

In this study, we have confirmed that a bacterial fingerprint depends of the growing condition as medium, temperature or other environmental parameters. A single variation in the culture medium, i.e. the concentration of heavy metals, involved a modifications of spectral fingerprint obtained by MALDI-tof (fig. 3.4, 3.5, 3.6).

The fingerprint of *Ralstonia metallidurans* CH34 growing in presence of gluconate was very different of its in presence of copper (0.85mM) or zinc (2mM) and Nickel (2mM). Moreover, the spectral modifications were different in function of the heavy metals. Interesting, in the fingerprint a periplasmic protein (cop K) induced in presence of copper (ions corresponding to the ratio m/z 8275.962) was observed. The identification of the copK in the fingerprint was realized by comparison of the molecular weight against the genome. It's very interesting to observe the presence of other entities that are over-expressed in presence of copper and Zinc (entities corresponding to ions characterized by a ratio m/z 1953 and 1542). The nature and the identification of these different entities are unknown.

Moreover, the expression of the copK was measured in function of the concentration of copper in the growth medium. For this study, the experimental protocol was adapted to observe periplasmic proteins. Bacteria were resuspended in 200ul of water (milli-Q) and were spotted on the MALDI plate (2 µl by sample). The samples were then dried at 37°C during 1h and 1µl of ethanol was added and dried at room temperature. After this step, 2µl of MALDI-tof Matrix solution (alpha2) were added and dried at room temperature. Twelve target wells were used for each organism to be entered into the database.

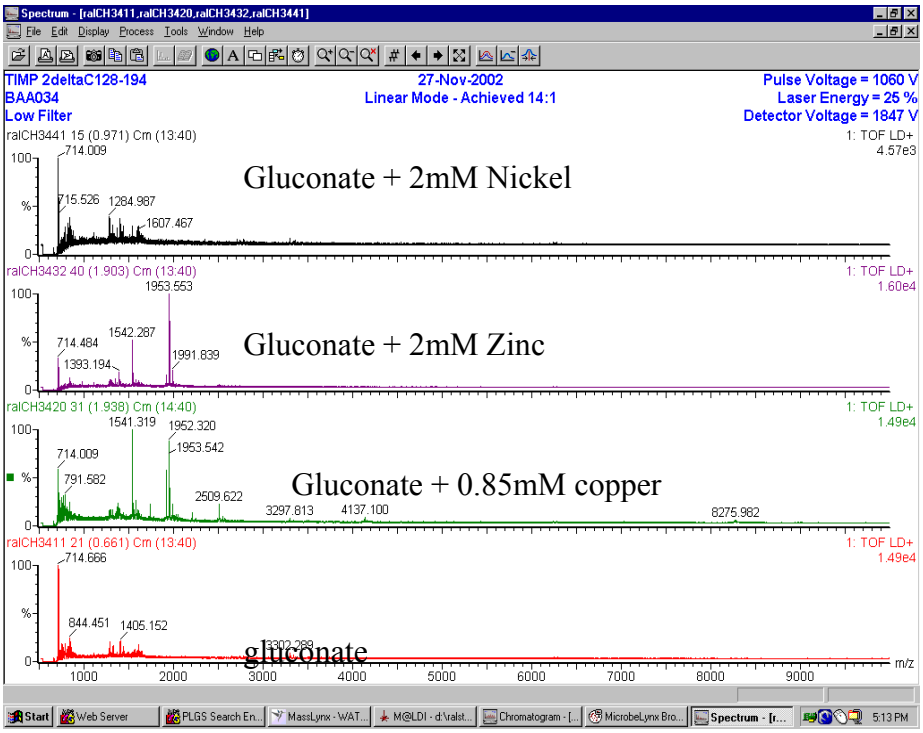


Fig. 4 MALDI-tof fingerprint of *Ralstonia metallidurans* CH34 in presence of different heavy metals

After the ethanol treatment (for destabilization of the bacterial membrane and the subsequent increase of the ionization of the surface and internal proteins), an other periplasmic protein (COP C) induced in presence of copper (0.85mM) was also observed. After an ethanol pre-

treatment, we can also observe the presence of COP K in zinc medium. This result is similar to this obtained by the 2-DE technique. These results are in preparation to publish in an international journal "Rapid communications in mass spectrometry" (Periplasmic protein expression of *Ralstonia metallidurans* induced by heavy metals : Intact cell MALDI-TOF study. 2003., Ruddy Wattiez, Larissa Hendrickx, Max Mergeay and Paul Falmagne, in preparation).

This approach will used to study environmental stress as temperature variation, oxidative stress in different MELiSSA strains as *Arthrospira* sp. PCC8005 and *Rhodospirillum Rubrum* ATCC25903.

Periplasmic proteins that are expected to be found are heat shock proteins as HSP70. These are up-regulated and have a extracellular, periplasmic and cytoplasmic localization in stressed conditions as temperature variation and oxidative stress.

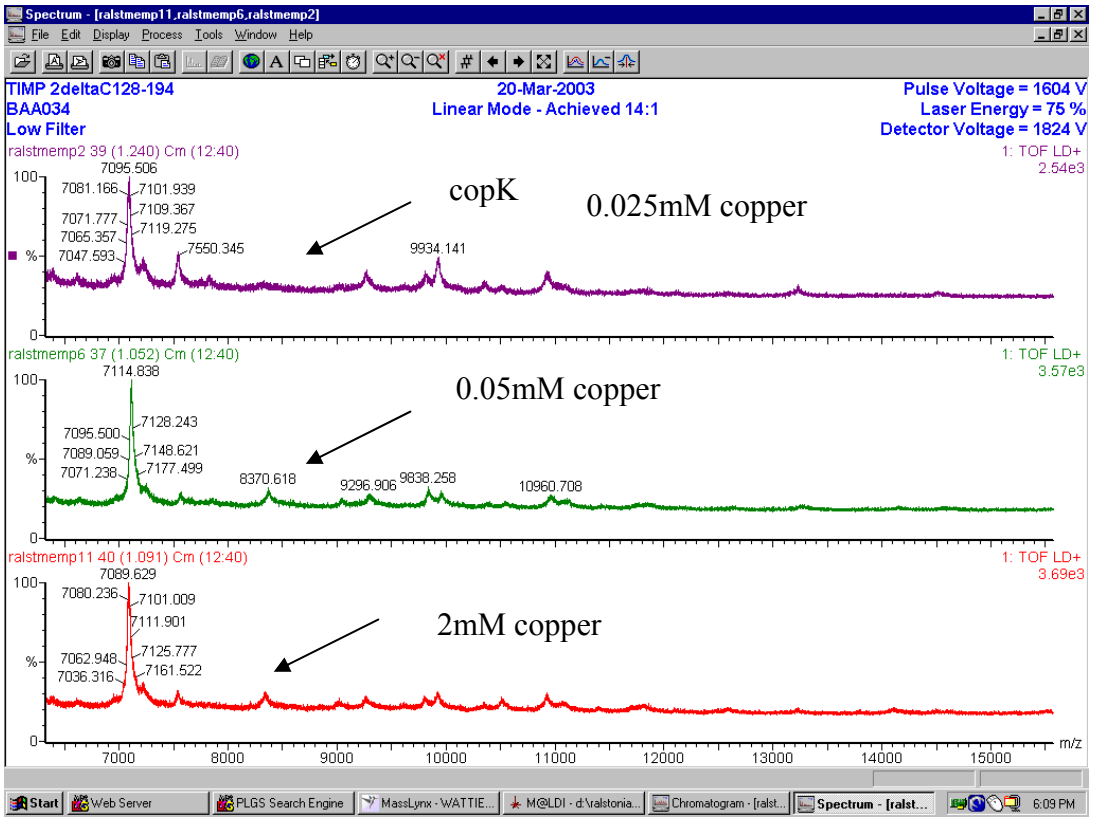


Fig. 3.5. MALDI-TOF fingerprint of *Ralstonia metallidurans* CH34 in presence of different concentrations of copper

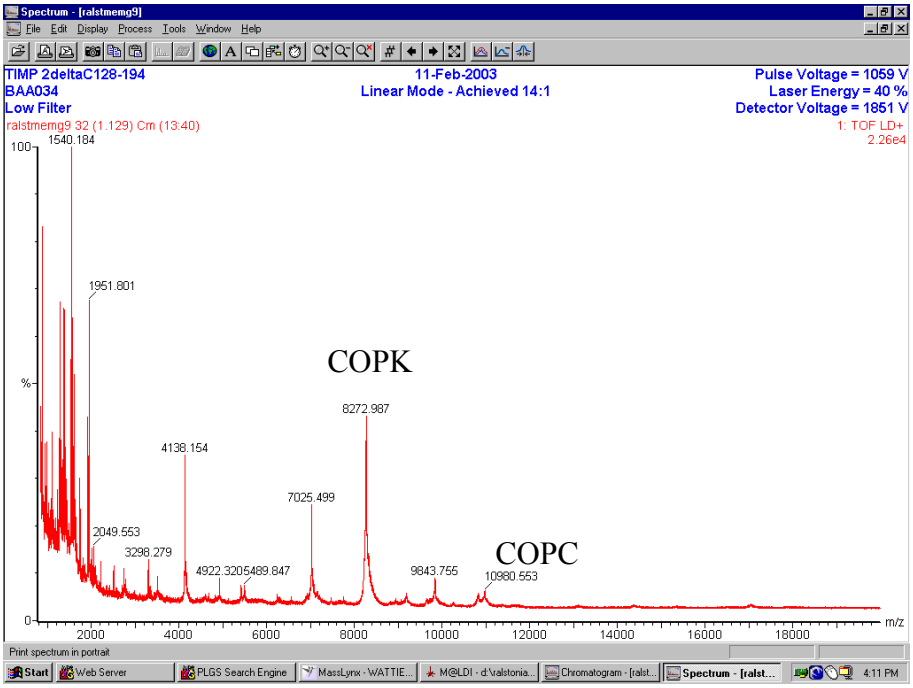


Fig. 3.6. MALDI-tof fingerprint of *Ralstonia metallidurans* CH34 in presence of copper. Presence of periplasmic protein COP C

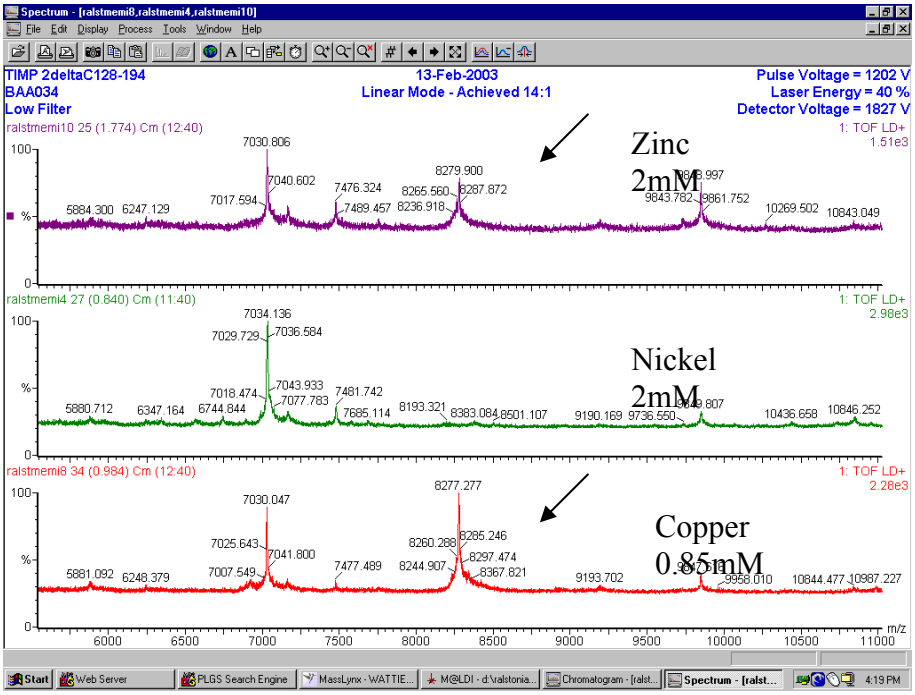


Fig. 3.7. MALDI-tof fingerprint of *Ralstonia metallidurans* CH34 in presence of copper, zinc or nickel.

3.5 *MALDI-TOF fingerprint of Arthrospira sp. and Rhodospirillum Rubrum: prelude of stress and axenicity studies*

In this study, a bacteria fingerprint database containing specific bacterial fingerprint of *Arthrospira* sp. PCC8005 and *Rhodospirillum rubrum* ATCC25903 was build.

As shown the figures 3.8. and 3.9., specific fingerprints of both bacteria were obtained just after centrifugation without prewashing step to avoid possible modifications of the bacterial surface. In this context, 96 different cultures (3ml) of both bacteria were analysed by the MALDI-TOF and a average mass spectral fingerprint obtained by the Microbiolynx algorithm was introduced in the database To test the pattern recognition algorithm (microbelynx ^{TM}), samples of both bacteria were submitted to the intact cell MALDI-TOF analyse and compared against our new database. A significant matching (100% probability) was observed with the respective specific fingerprint of both bacteria. As shown for *Ralstonia metallidurans*, our system is able to identify systematically these both bacteria by comparison with our bacteria fingerprint database (Microbiolynx ^{TM} algorithms).

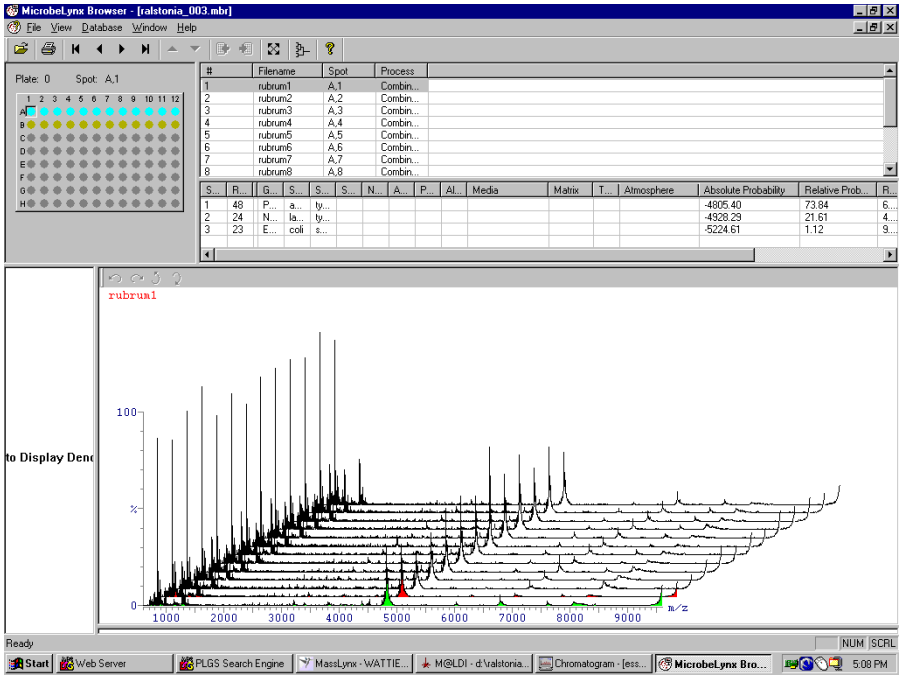


Fig. 3.8. MALDI-TOF fingerprint of *Rhodospirillum rubrum* ATCC25903

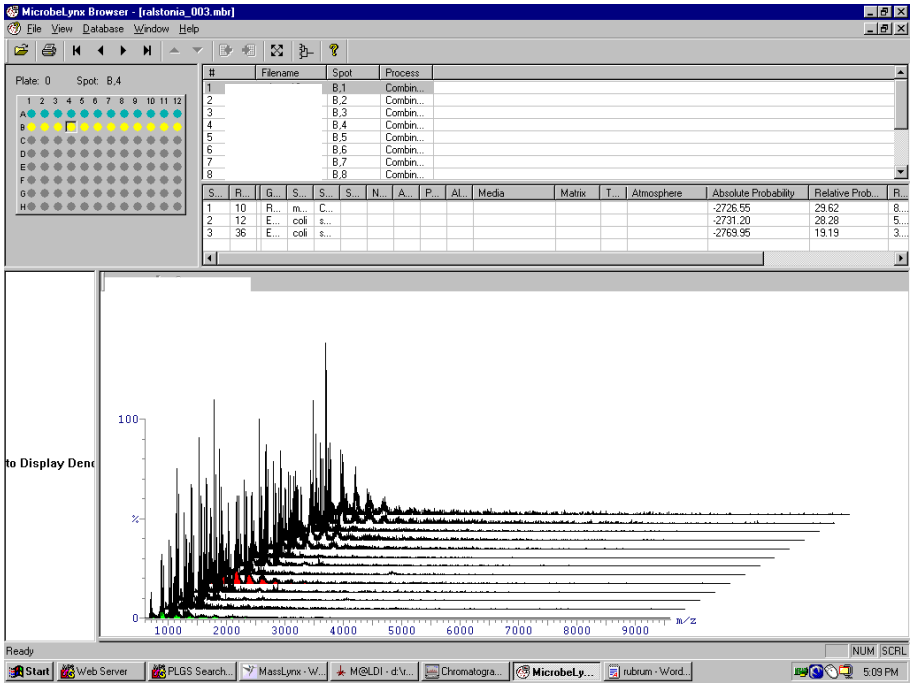


Fig. 3.9. MALDI-TOF fingerprint of *Arthrospira* sp. PCC8005

3.6 Conclusions

Intact cell MALDI-TOF-MS proved to be a powerfull analytical method, capable of real-time detection of changes in environmental conditions and sub-typing of bacteria. Mass-Fingerprinting proved to be very rapid. The entire process from sample preparation to result took only about 1 minute for each test microorganism. Mass-fingerprints of the model organism *R. metallidurans* CH34, *R. rubrum* ATCC25903 and *Arthrospira* sp. PCC8005 could be reliably matched against databases of quality controlled reference mass spectra resulting in unique physico-chemical fingerprint for the species tested. Changes in environmental conditions changed the fingerprint, due to differentially expressed proteins expressed on the surface of the bacteria.

Further research will concentrate on the detection of the effect of stress on the MELiSSA organisms and monitoring axenicity in *R. rubrum* or *Arthrospira* sp. cultures using MALDI-TOF-MS fingerprinting.

4 PCR-BASED GENOMIC ANALYSIS OF MICROBIAL SAMPLES

4.1 Introduction

The polymerase chain reaction (PCR) method can be described as exponentional amplificantion of very small amounts of nucleic in a highly specific manner using DNA basepair pairing (hybridisation) between short oligonucleotide probes and target sequences within the chromosome. More information about the principles of the Polymerase Chain Reaction (PCR) can be found in detail in TN70.8 (section 3). To investigate whether PCR based approaches can be used to monitor axenicity in the MELiSSA loop, we decided to focus on DNA targets encoding small ribosomal RNA (16S rRNA). This approach had various advantages. First, rRNA genes contain highly conserved as well as highly variable regions, allowing unambiguous identification. Second, large databases of 16 rRNA gene sequences exist permitting computational design of specific primers through sequence comparison.

4.2 Materials and methods

4.2.1 STRAINS AND DNAS.

Ralstonia metallidurans strain CH34 and *Rhodospirillum rubrum* strain ATCC 11170 were obtained from M. Mergeay, SCK-CEN, Mol as live cultures. *Ralstonia* and *Burkholderia* chromosomal DNAs were obtained from J. Goris, Universiteit Gent, Gent. The following strains were used (strain collection numbers taken from the Laboratory of Microbiology, Ghent; T is typestrain): *Burkholderia cepacia* (1222^T), *Burkholderia graminis* (18924^T), *Ralstonia basilensis* (18990^T), *Ralstonia campinensis* (19282^T), *Ralstonia gilardii* (5886), *Ralstonia mannitolilytica* (6866^T), *Ralstonia metallidurans* CH34 (1195^T), *Ralstonia metallidurans* CH34 (18526^T), *Ralstonia metallidurans* CH79 (19296), *Ralstonia paucula* (3244^T), *Ralstonia pickettii* (5942^T), and *Ralstonia solanacearum* (2299^T).

4.2.2 GROWTH OF BACTERIAL CULTURES.

R. metallidurans was aerobically grown in a small erlenmeyer flask using Luria Broth supplemented with 2 mM $ZnSO_4$ and 1 mM $NiCl_2$. Growth proceeded at a 28°C shaker incubator for 15 hrs or more until at least late exponential stage was reached. *R. rubrum* was grown in a 15 mL Falcon tube using Melissa medium (TN70.1) for 4 weeks at room temperature placed under a fluorescent tube (PHILIPS ® 30W/83) with a light flux of about $20W/cm^2$.

4.2.3 PREPARATION OF GENOMIC DNA.

R. metallidurans and *R. rubrum* genomic DNA (gDNA) was prepared essentially using the Pitcher method (REF). Cells were harvested directly from an overgrown petri-dish. In general, a full loop of cells was transferred to 100 μL of TE (10 mM Tris, 1 mM EDTA pH 8.0) and cells were immediately centrifuged down and resuspended in 500 μL GES (5M guanidine thiocyanate [Sigma G-6639], 1% w/v sarkosyl [Sigma L-9150], 0.1 M EDTA, pH 8.0). After 5-10 minutes on ice, 250 μL of 7M NH_4Ac was added, mixed, and the mixture was left on ice for a further 10 min. Subsequently, 500 μL of chloroform:iso-amylalcohol (24:1) was added, mixed well, and the organic phase was separated by centrifugation. Supernatans (700 μL) was taken and mixed with 380 μL of iso-propanol for precipitation of nucleic acids. The resulting pellet was washed twice with 70% ethanol and finally dissolved in 100 μL Milli-Q water. RNAs were degraded by adding 10 μL DNAse-free RNAse (500 μg/mL) and incubation at 37 °C for 90 min. The re-precipitated gDNA was finally dissolved into 100 μL $T_{0.1}E$ (10 mM Tris, 0.1 mM EDTA pH 8.0) and stored at –20 °C until use. Addition of EDTA is a precaution against endonucleases, as EDTA is a strong metal-chelating reagent and divalent ions such as Mg++ are often needed for enzymatic activity. The rationale to use 0.1 mM EDTA instead of 1 mM EDTA is that an excess of EDTA may also result in chelation of the divalent metal ions during the PCR, e.g. the DNA polymerase would not work properly. Therefore, 10x less EDTA is used, still chelating metal ions, thereby inhibiting endonucleases to some extent while avoiding too much interferance with Taq DNA polymerase activity. Genomic DNA from *Arthrospira platensis* was prepared by a slight modification of the above procedure by washing the harvested *A. platensis* cells twice with 100% ethanol and twice with RS buffer (150 mM NaCl, 10 mM EDTA, pH 8.0) followed by a pretreatment for 30 min at 37 °C with 150 μL lysozyme (50 mg/mL) and a further 60 min at 37 °C with 25 μg Proteinase K. Cell lysis was then proceeded with GES as described above. Genomic DNAs were routinely checked for purity and integrity by electrophoresis on 1% agarose gels.

4.2.4 PRIMER DESIGN.

The guidelines for proper primer design can be found in TN70.8 (section 3.5) and the actual procedures that were followed were as described in TN70.9 (section 1). The resulting sequences of the oligonucleotide probes (PCR primers) are listed in Table 4.1. Further sequence details can be found in addendum B of TN70.9. In total, four primer pairs were used (Fig. 4.1.). Much care was taken in choosing primers with similar optimal annealing temperatures and the target location was choosen in such a way that the amplicon size would be characteristic for each given primer pair. This allows for proper discrimination of the various amplicons when they are electrophorised.

code	target	5'-3' sequence	length	GC%	Tm
EUB338F	ACTCCTACGGGAGGCAGC	ACTCCTACgggAggCAgC	18	66,7	55
EUB338R	ACTCCTACGGGAGGCAGC	gCTgCCTCCCgTAggAgT	18	66,7	55
UNIV1390F	TTGTACACACCGCCCGTC	TTgTACACACCgCCCgTC	18	61,1	53
UNIV1390R	TTGTACACACCGCCCGTC	gACgggCggTgTgTACAA	18	61,1	53
RRUB999R	CGTGACACTTCCAGAGAT	ATCTCTggAAgTgTCACg	18	50	54

RMET110F	CATTGGAACGTACCCTGT	CATTggAACgTACCCTgT	18	50	54
APLA1759R	AGTTGGGGTGAGGTAGTC	gACTACCTCACCCCAACT	18	55,6	54

Table 4.1.: species specific and universal primers

4.2.5 TESTING OF PRIMER SPECIFICITY.

Each of the three species-specific primer pairs was tested for specificity under increasing stringency, i.e. using annealing temperatures of 50 ºC, 52 ºC, 54 ºC, and 56 ºC. At the highest annealing temperature, each species-specific primer pair was tested against the two other gDNAs, e.g. primer pair RMET110F /EUB338 was checked against not only *R. metallidurans* gDNA, but also against the gDNAs of *R. rubrum* and *A. platensis*, and soforth. Finally, 16S rDNA primers for *R. metallidurans* were checked for specificity at the species level by applying the RMET110F /EUB338 pair to the gDNAs of 11 *Ralstonia* species and one *Burkholderia* species, using highly stringent annealing temperatures (56 ºC, 60 ºC, 64 ºC, and 68 ºC). All PCR reactions were otherwise performed with the same PCR reaction conditions as outlined below.

4.2.6 EXPERIMENTAL SET-UP OF THE 'SPIKING'.

Spiking experiments were performed by artificially contaminating a full grown culture of *R. rubrum* and *R. metallidurans* with serial dilutions of *R. metallidurans* and *R. rubrum*, respectively. A first preliminary experiment used ten-fold serial dilutions, whereas a refined second spiking experiment used four-fold serial dilutions (see text and Fig. 2.). Each experiment had two controls (one of each pure culture, also noted as 'carrier') and a number of contaminations (also noted as 'spikes'), each with a decreasing number of contaminating cells. Each spike contained 100 μL carrier cells and 100 μL contaminating dilution. Controls contained 100 μL carrier cells and 100 μL of medium used to grow the other strain. Cells in the controls and spikes were harvested by centrifugation and gDNA was prepared as describe above except that the DNA pellet was finally dissolved in 1 mL MQ-H_2O. Of this, 4 μL was used for PCR. The *R. metallidurans* and *R. rubrum* specific primer pairs were applied on these DNAs in separate PCRs, but reaction samples were co-migrated in the same well of the 1% agarose gell during electrophoresis (see text). All PCR reactions were performed with the same PCR reaction conditions as outlined below.

4.2.7 PCR REACTIONS.

All reactions were performed in a 20 μL reaction volume using the following constant conditions: 0.2 mM dNTPs, 50 ng of each primer, 1x PCR buffer (1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 0.01 % stabiliser), 0.8 U *Thermus aquaticus* DNA polymerase (SuperTaq, HT Biotechnology, UK). Unless otherwise stated, the following cycling profile was used: 1 cycle of 3 min at 94 ºC, 34 cycles of 45 s at 94 ºC, 45 s at 56 ºC, 45 s at 72 ºC, and one cycle of 7 min at 72 ºC.

4.2.8 GEL ELECTROPHORESIS.

Samples (typically 8 μL) were mixed with 2 μL loading dye (6x buffer; 0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll) and electrophoresed on a 1% agarose gel using 1x TAE buffer (50x; 0.05 M EDTA, 2M Tris-Acetate, pH 8.0). Gels were stained in a ethidium bromide bath (20 μg/mL) for 10-15 seconds and rinsed with water. DNA was visualised using a 254 nm UV box.

4.3 Results and discussion

We designed species specific primers for *R. metallidurans*, *R. rubrum*, and *A. platensis* by using the ARB software as explained in TN70.9 (section 1). These primers were thoroughly checked by *in silico* analysis for their specificity using the NCBI nrdb and RDP databases of 16S rRNA sequences (see TN70.9). We also made use of existing universal primers that are known to bind to conserved regions within the 16 rRNA gene. By combining the specific primers and the universal primers, species-specific primer pairs could be obtained (Fig. 4.1.)

Care was taken to design the primers in such a way that amplicons could be clearly discerned from each other by size. In a first experiment, primer pairs were tested at 4 different annealing temperatures, 50 ºC, 52 ºC, 54 ºC, and 56 ºC (Fig. 2). For the three species-specific primer pairs, amplicons with the expected size were obtained for all four temperatures (using the target genomic DNA).

Fig. 1: Layout of species-specific primer pairs

However, at the lowest temperature, aspecific priming occurred for *A. platensis* (lane a, Fig. 4.2.). The universal primer pair was only tested at 50 ºC, and aspecific priming could be seen here as well for *R. metallidurans* and *A. platensis* (lanes M and A, Fig. 4.2.). Because of these results, we decided to use an annealing temperature of 56ºC for all further PCRs with these four primer pairs.

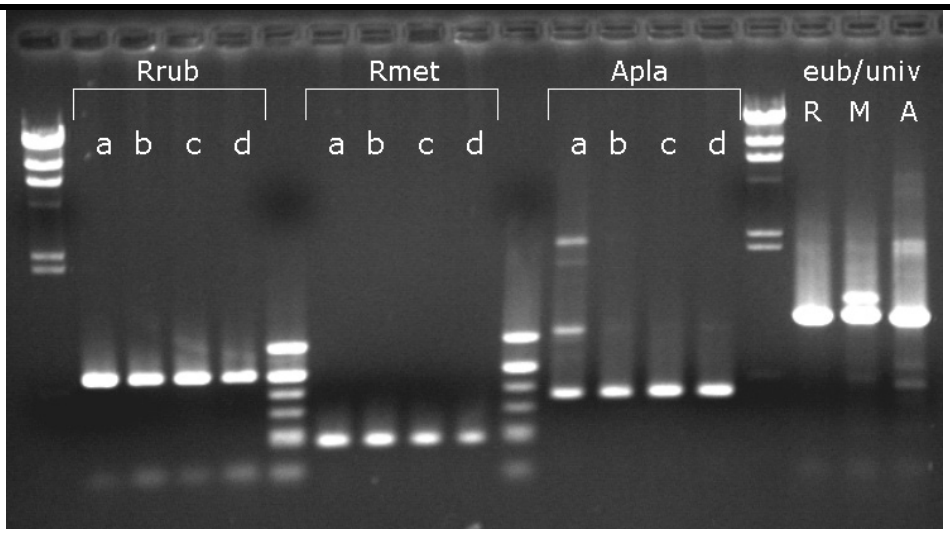


Fig. 4.2.: Testing out four annealing temperatures on genomic DNA of *Rhodospirilum rubrum* (Rrub), *Ralstonia metallidurans* (Rmet), and *Arthrospira platensis* (Apla), using 16S-specific primers as listed in Table 4.1. and according the layout in Fig. 4.1. [a] 50C, [b] 52C, [c] 54C, [d] 56C

Next, we tested the specificity of each of the species-specific primer pairs against the genomic DNAs of all three species, i.e. *R. metallidurans*, *R. rubrum*, and *A. platensis*. Very discrete signals (single sharp band of expected size) were obtained for the corresponding target genomes (Apla[a], Rmet[b], Rrub[c], Fig. 4.3), whereas for non-target genomic DNA, no signal was obtained (Apla[c], Rmet[ac], Rrub[ab], Fig. 4.3) except for primer pair RMET110F / EUB338R against *A. platensis* gDNA, where 2 very faint bands (white arrows) of aberrant size were observed. As expected, the universal primer pair resulted in strong discrete signals for all three gDNAs (lane d, Fig. 3).

To further verify the proper design of the PCR primers and in an attempt to assess their discriminative power we subjected the gDNAs of seven related *Ralstonia* and two *Burkholderia* strains to the *R. metallidurans* primer pair (`RMET110F/EUB338R`). Two additional strains of *R. metallidurans* were also tested (see Materials & Methods). The phylogeny of these strains was previously studied with a range of methods (Goris *et al.*, 2001)[1]. The target sites for the RMET110F primer in the 16S rRNA genes of these related organsims differ in up to 4 nucleotides from the *R. metallidurans* CH34 target site (Fig. 4.4). The corresponding regions of the *A. platensis* and *R. rubrum* 16S rRNA genes are also given. Whereas the *A. platensis* site clearly deviates from the *R. metallidurans* target site with 11 nucleotides, the latter differs in only four nucleotides, as many mismatches as for the *B. cepacia* target site. The fact that *R. rubrum* gDNA does not give any signal at the least stringent condition (Fig. 4.3) illustrates very well the effect of mismatches at the 3'-end of the binding site.

[1] Int. J. Syst. Evol. Microbiol. (2001)51:1773-82.

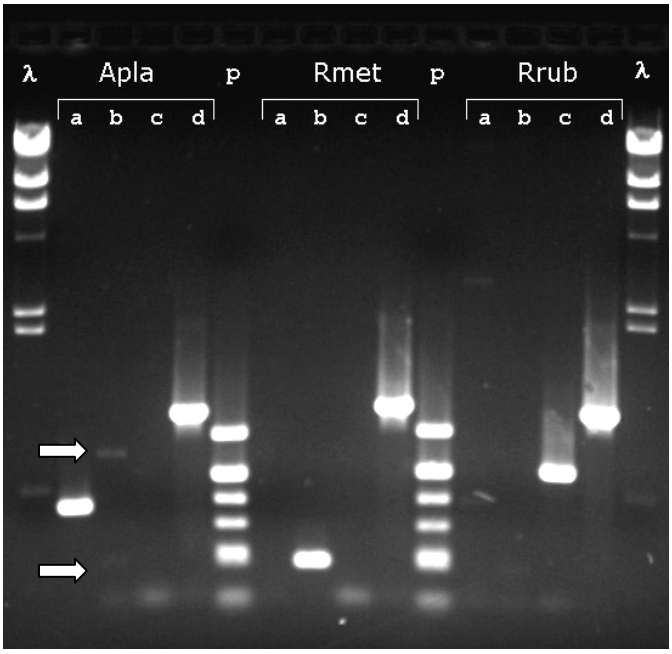


Fig. 4.3: testing out species specificity (a = UNIV1390F / APLA1759R ;b = RMET110F/EUB338R ;c = EUB338F/RRUB999R ;d = EUB338F/UNIV1390); Whilte arrows indicate aspecific amplification on *Arthrospira* sp. DNA

```
Rmet    CATTGGAACGTACCCTGT     -
Rgil    YATCGRAACGYSCCCTGT    ?/18
Rpau    CATCGGAACGTACCCTGT    1/18
Rsol    CATCGGAACGTGCCCTGT    2/18
Rbas    CATCGGAACGTGCCCTGT    2/18
Rcam    CATCGGAACGTGCCCTGT    2/18
Bgra    CATCGGAACATGTCCTGT    4/18
Bcep    CATCGGAACATGTCCTGT    4/18

Rrub    CGTGGGAACGTACCTTGG    4/18
Apla    CGTGAGAATCTGGCTCCC   11/18
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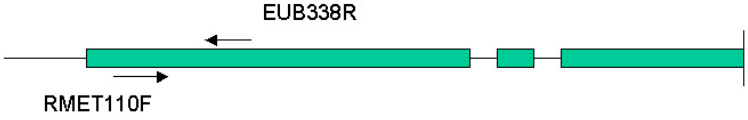

Fig. 4.4: RMET110 target sites in the 16S rRNA gene of various organisms

PCRs with RMET110F/EUB338R were performed with increasing stringency, i.e. at four different annealing temperatures: 56 ºC, 60 ºC, 64 ºC, and 68 ºC. The first increase to 60 ºC had little effect on the strength of the amplification signal, but at 64 ºC amplification signals were strongest for the *R. metallidurans* and the closely related species *R. paucula*, whereas the more distant related species *R. solanacearum*, *R. campinensis*, and *B. graminis* clearly displayed weaker signals (Fig. 4.5).

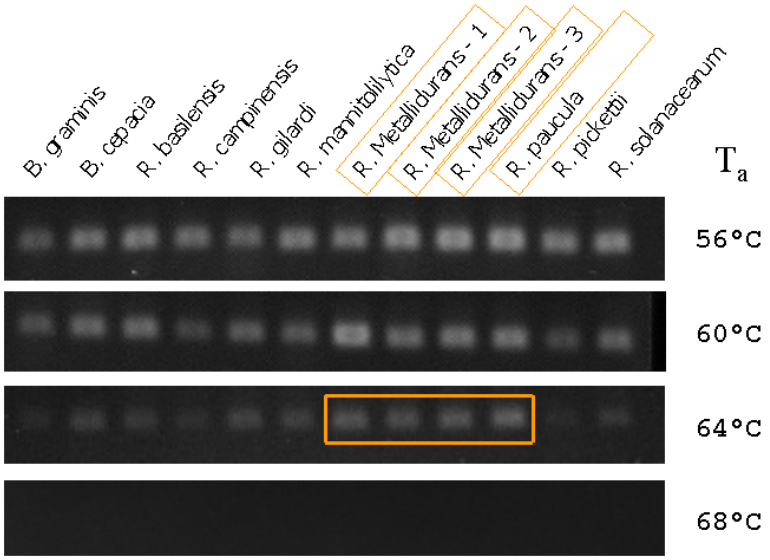

Fig. 4.5: primer specificity at the species and genus level

These differences in signal strength was reflected in the number of mismatches between the RMET110F primer and the target sites for these organisms (Fig. 4.4). At the highest stringency (68 ºC), no signal was obtained for any of the gDNA's. Obviously, at this temperature, at least one primer of the pair RMET110F/EUB338R does not anneal properly. Such high annealing temperatures can only be tested by adjusting the salt concentration (not yet done). Clearly, discrimination between *R. metallidurans* and other *Ralstonia* species and related organisms within the same order was more dificult with this primer pair.

4.4 Conclusion

The primers that were designed for the detection of *A. platensis*, *R. rubrum*, and *R. metallidurans* are sufficiently specific in the context of the MELiSSA reactor. At an annealing temperature of 56 °C, no crosshybridisation could be observed (e.g. no amplicon could be seen for the *R. metallidurans* primers applied at *A. platensis* or *R. rubrum* gDNA, and soforth), although higher annealing temperatures could be used to enhance specificity. This PCR-based study is a case study, in which possible contaminants originating from other compartments, in particular any of the principle reactor strains, is being studied. We agreed early on to test the specificity of PCR primers on Apla, Rrub and Rmet, as these strains can be cultured readily and from which genomic DNA could be prepared. Our study shows clearly that our PCR -

primer pairs are highly specific, in particular on an elevated annealing temperature, and that thus PCR can be used to detect intercompartment contamination by reactor strains. In theory, this can be done equally well on the other 2 principle reactor strains, given culturability and gDNA availability, as well as on every organism of C1, given availability of consortium composition. Evidently, monitoring of external contamination would require a more general approach, like FCM, or possible with adapted PCR technologies (i.e. mediated by PNA blocking).

5 OPTIMISATION OF THE HORIZONTAL GENE TRANSFER PROTOCOL IN *RHODOSPIRILLUM RUBRUM*

5.1 Introduction

The *R. rubrum* ATCC25903 compartment is residing downstream of the first compartment in the MELiSSA loop, which harbors a consortium of unknown bacteria. Leakage from the first compartment to the second would not only result in contamination of the second reactor, it could also involve possible genetic exchange between the contaminant and *R. rubrum*.

In the literature it has already been noticed that *R. rubrum* ATCC25903 can receive plasmids via conjugation (Saegesser et al., 1992; Ghosh et al., 1994). In this section, a mating protocol was designed for mating experiments with *R. rubrum* ATCC25903.

5.2 The mating partners

The mating partners are the *E. coli* strains CM404, CM1962, CM2034, CM238, CM1045, the *R. metallidurans* strain AE104 and *R. rubrum* ATCC25903.

Here follows a brief description of the strains:

E. coli CM404
Parent = HB101; rpsL; leu^-, pro^-, $thia^-$, lac^-
Plasmid = pRK2013 (Tra^+, Km^r, Nm^r)

E. coli CM1962
Parent = DH10B, rpsL (Sm^r); Ara^-, Leu^-, Gal^-
Plasmid = pMOL222 (Tra^-, Mob^+; Amp^r, Km^r, Ni^r; IncQ) (pKT240:ncc-nte-Km100, NiR) (derivative of pKT240; ~27 kb)

E. coli CM2034
Parent = S17-1 (Sm^r, Sp^r, Tm^r; Pro^-; Tra^+)
Plasmid = pMOL222 (Tra^-, Mob^+; Amp^r, Km^r, Ni^r; IncQ) (pKT240:ncc-nte-Km100, NiR) (derivative of pKT240; ~27 kb)

E. coli CM238
Parent = M5020Gδcar (Pro^-, Uri^-, Arg^-) rpsL (Sm^r)
Plasmid = pZU8 (RP4 derivative; Tra^+, Mob^+; Km^r, Tet^r, Hg^r; ::Mu18A1{amp})

R. metallidurans AE104
Parent = CH34
Plasmid = none

R. rubrum ATCC25903
Wild type

5.3 Testing growth of the different mating partners

Before mating experiments were performed, the mating partners needed to be tested on their ability to grow on different selective media:

Growth tests in aerobic dark conditions (7days, 30°C):

Medium	*R. rubrum*	AE104	CM404	CM1962	CM2034	CM238	CM1045
869	+	+	+	+	+	+	+
869 Ni5	-	-	-	+/-	-	-	-
869 Ni5Sm20	-	-	-	+/-	-	-	-
869 Tell 0.5	+	+	-	+/-	+/--	-	+/--
869 Tell 1	+	+/-	-	+/--	-	-	-
869 Tell 2	+	-	-	-	-	-	-
869 Tell 5	-	-	-	-	-	-	-
869 Km50	-	+	+	+	+	+	+
869 Km50Sm20	-	+/-	+	+	+	+	nd
869 Km75Sm50	-	-	+	+	+	+	nd
Sistrom	+	+	-	+/--	-	-	-
Sistr Tell 0.5	+	+/-	-	-	-	-	-
Sistr Tell1	+/-	+/--	-	-	-	-	-
Sistr Tell2	-	-	-	-	-	-	-
Sistr Tell5	-	-	-	-	-	-	-
Sistr Km50	-	+	-	+/--	-	-	-
Sistr Km75	-	+	-	-	-	-	-
284	-	+	-	-	-	-	-
284 Gluc	-	+	-	-	-	-	-
284 Gluc Ni 5	-	-	-	-	-	-	-

Table 5.1. Growthtest on different selective media of the different mating partners

Mating procedures, as described by Saegesser *et al.* (1992) were used as a basis to design the mating experiments.

5.4 Mating procedure according to Saegesser et al. (1992):

5.4.1 MATING PROCEDURE

5ml of the *E. coli* donor and helper strain were grown to an OD_{660} of approx. 0.8-1.5 at 37°C in LB medium. Cells (1ml) were washed twice by centrifugation with the same volume of a sterile salt solution. Chemotrophically grown cells of *R. rubrum* were harvested at an OD660 of approx 1-1.5, pelleted and then resuspended in the same volume of the sterile salt solution.

For triparental mating, $2X10^8$ cfu of chemotrophically-grown cells of *R. rubrum* in a salt solution were mixed with the donor *E. coli* and the helper *E. coli* at a ratio of 100:1:1. The mating mixture was pelleted and resuspended in 20µl saltsolution and plated immediately onto solid Sistrom medium supplemented with 3% peptone and 1% yeast extract. The plates were allowed to dry for 30-45 min at RT and then incubated for 6h aerobically in the dark at 30°C.

The selection of the transconjugants was performed by streaking the cells from the conjugation plates onto Sistrom plates containing the selective concentrations of the proper antibiotics. Counterselection was provided by amino acid-auxotrophy of the *E. coli* used. Incubation was performed either chemotrophically or phototrophically at 30°C for 5 days.

After incubation the conjugation frequency was determined as the ratio of the number of transconjugants observed with antibiotic selection to the total number of viable *R. rubrum.*

5.4.2 GROWTH OF BACTERIA

R. rubrum was grown at 30°C using Sistrom minimal medium A (without casamino acids) (Sistrom, 1960) aerobically in the dark, or anaerobically in the light. Growth on solid medium was performed using Sistrom minimal medium A supplemented with 1.5% agar.
Cultures of *E. coli* were maintained in LB medium with the proper selective antibiotics.

5.4.3 TESTING GROWTH IN ANAEROBIC/AEROBIC, LIGHT/DARK CONDITIONS ON 869 OR SISTROM IN HETEROTROPHIC CONDITIONS

The growth tests were done incubated at either 30°C or 22°C. In both cases we obtain the same results. Growth was inhibited when *R. rubrum* was grown on 869 in light conditions. Therefore it was decided to grow the mating and selection plates in dark heterotrophic conditions.

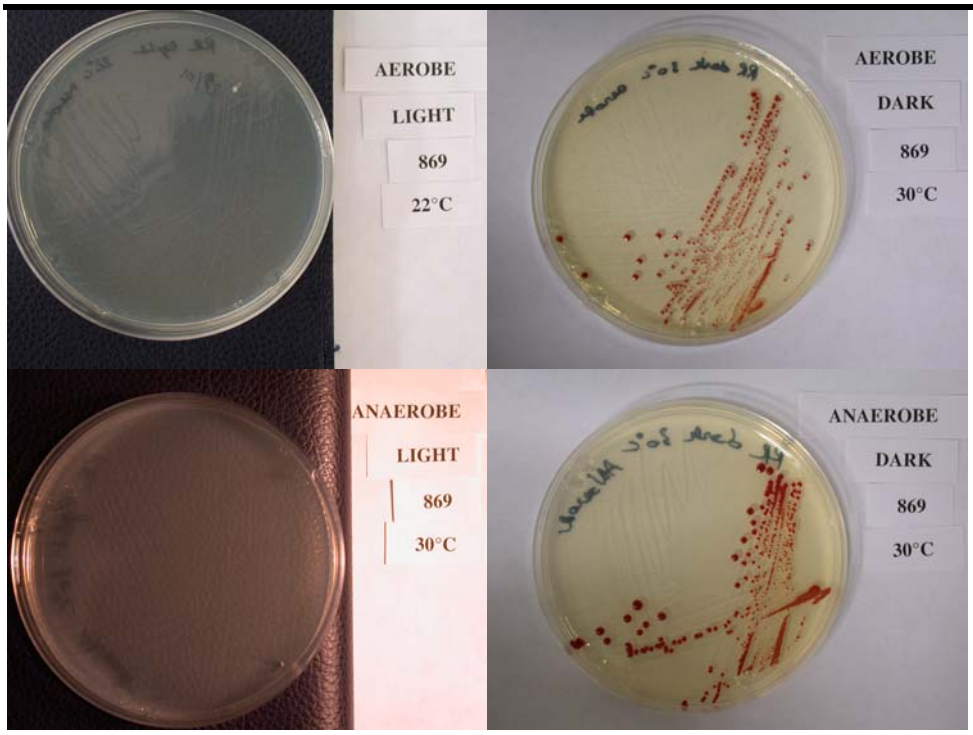

Fig. 5.1. Growth test on 869 medium.

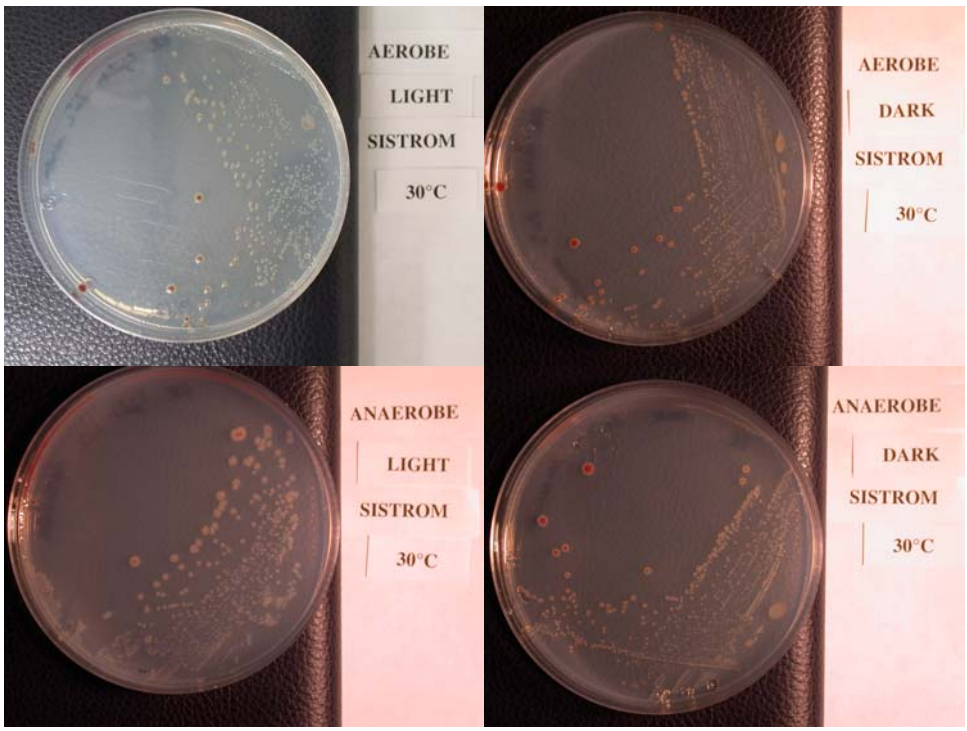

Fig. 5.2. Growth test on Sistrom medium.

5.4.4 EFFECT OF THE MATING PLATE

Two times, triparental matings between *E. coli* strains CM1962, CM404 en *R. rubrum* were performed using either the mating plate 869 or the mating plate Sistrom 3% peptone 1% yeast (SisPY).

Mating plate medium	CM1962 (cfu/ml)	CM404 (cfu/ml)	R. rubrum (cfu/ml)	Transconj. (cfu/ml)	T/D	T/R	Freq. false pos
869	5.9^{8}	7.5^{9}	8.3^{6}	1.1^{2}	1.8^{-7}	1.3^{-5}	$<1.3^{-6}$
SisPY	4.3^{8}	3.1^{10}	4.2^{6}	8.4^{2}	1.9^{-6}	2^{-4}	7.8^{-6}

The mating on 869 seems less efficient then mating on SisPY plates.

5.4.5 EFFECT OF THE *R. RUBRUM* PREINCUBATION DROP

A triparental mating between *E. coli* strains CM1962, CM404 en *R. rubrum* was performed using either a preincubation of *R. rubrum* on the mating plate (SisPY) or together with the other mating partners.

Preincubation	CM1962 (cfu/ml)	CM404 (cfu/ml)	*R. rubrum* (cfu/ml)	Transconj. (cfu/ml)	T/D	T/R	Freq. false pos (CM1962)
1 day	1.2^{9}	2.3^{8}	1.4^{8}	1.75^{4}	1.5^{-5}	1.3^{-4}	$<9.3^{-7}$
0 day	4.3^{9}	3.1^{10}	4.2^{6}	8.4^{2}	1.9^{-7}	2^{-4}	$<2.6^{-6}$

There is an advantage observable in the detection limit of the frequency of false positives (colonies miscalculated as transconjugants). Hence, it is advisable to incubate *R. rubrum* one day earlier on the mating plate.

5.4.6 EFFECT OF THE MEDIUM OF THE *R. RUBRUM* SUSPENSION BEFORE MATING

A triparental mating between *E. coli* strains CM1962, CM404 en *R. rubrum* was performed using either a suspension of *R. rubrum* precultivated on MELiSSA medium, 869, 869 Tell 2mM, Sistrom, Sistrom Tell 1mM. *R. rubrum* was preincubated on the mating plate (SisPY) one day ahead.

Culture	CM1962 (cfu/ml)	CM404 (cfu/ml)	*R. rubrum* (cfu/ml)	Transconj. (cfu/ml)	T/D	T/R	Freq. false pos
MELiS	3.5^{11}	5.3^{9}	3.6^{6}	3.24^{2}	9.2^{-8}	9^{-5}	$<3.1^{-6}$
869	1.2^{9}	2.3^{8}	1.4^{8}	1.75^{4}	1.5^{-5}	1.3^{-4}	$<1.3^{-8}$
869 Tell	2.5^{9}	5.2^{9}	3.2^{6}	1.11^{2}	4.4^{-6}	3.4^{-5}	$<2.9^{-6}$
Sis	5.6^{10}	6.3^{7}	1.9^{7}	2.04^{3}	3.6^{-6}	1.1^{-4}	$<1.7^{-7}$
Sis Tell	6.4^{10}	9.4^{6}	4.1^{6}	3.84^{2}	6.0^{-6}	9.4^{-4}	$<3.7^{-6}$

When *R. rubrum* was grown on MELiSSA medium or 869 Tell, the conjugation frequency was lower. Using other media, higher frequencies were observed, where growth in Sis Tell gave rise to the highest frequency. However, when *R. rubrum* needed to grow in Sis Tell, an incubation time of more than 7 days was necessary. Hence it was chosen to grow *R. rubrum* on Sistrom medium in future experiments, because *R. rubrum* could grow relatively quick under relatively selective conditions.

5.4.7 EFFECT OF THE SELECTIVE MEDIA AFTER MATING:

A triparental mating between *E. coli* strains CM1962, CM404 en *R. rubrum* was performed using a suspension of *R. rubrum* precultivated on Sistrom medium. *R. rubrum* was preincubated on the mating plate (SisPY) one day ahead. Selection of transconjugants were performed with Sistrom Km 50, Sistrom Tell 0.5 mM Km 50, 869 Tell 2 mM Km 50. As a control the same selective media were used for the mating between CM1962 and *R. rubrum*. In the following table the number of colony forming units of the mating partners CM1962, CM404 and *R. rubrum* found were respectively 1.0^{11} cfu/ml, 2.0^{12}cfu/ml and 3.7^{11}cfu/ml.

Transcon-jugants were selected on:	Transconj. (cfu/ml)	T/D	T/R	Freq. false pos
Sistrom Km	6.8^{7}	6.8^{-4}	1.8^{-4}	$<3.2^{-11}$
Sistrom Tell 0.5 Km50	1.2^{7}	1.2^{-4}	3.2^{-5}	$<3.2^{-11}$
869 Tell 2 Km 50	1.3^{7}	1.3^{-4}	3.5^{-5}	$<3.2^{-11}$
Sistrom Tel0.5 Km50 Amp100	4.1^{3}	4.1^{-3}	1.1^{-8}	$<3.2^{-11}$
869 Tell2 Km50 Amp100	2.0^{5}	1.9^{-5}	5.4^{-7}	$<3.2^{-11}$

The selective medium, that is used to count transconjugants resulted in a transferfrequency going from 1.8^{-4} to 1.1^{-8}. It seems that Sistrom Km50 would be the ideal medium to

select for *R. rubrum* transconjugants. However, in the control mating between *R. rubrum* and CM1962, without helper strain a background frequency of $5^{\wedge}10^{-8}$ was detected on Sistrom Km 50 and a backgound frequency of $4^{\wedge}10^{-8}$ was detected on 869 Tell 2mM Km50. On Sistrom Tell 0.5mM Km50 no background was detected.

5.4.8 REPRODUCIBILITY

A triparental mating between *E. coli* strains CM1962, CM404 en *R. rubrum* was performed using a suspension of *R. rubrum* precultivated on Sistrom medium. *R. rubrum* was preincubated on the mating plate (SisPY) one day ahead. Selection of transconjugants were performed with Sistrom Tell 0.5 mM Km 50.

Culture	CM1962 (cfu/ml)	CM404 (cfu/ml)	*R. rubrum* (cfu/ml)	Transconj. (cfu/ml)	T/D	T/R
1	$3.7^{\wedge}10^{9}$	$1.3^{\wedge}10^{9}$	$1.4^{\wedge}10^{8}$	$1.8^{\wedge}10^{4}$	$4.9^{\wedge}10^{-6}$	$1.3^{\wedge}10^{-4}$
2	$1.8^{\wedge}10^{9}$	$2.4^{\wedge}10^{8}$	$2.7^{\wedge}10^{8}$	$1.6^{\wedge}10^{4}$	$8.9^{\wedge}10^{-5}$	$5.9^{\wedge}10^{-5}$
3	$8.4^{\wedge}10^{8}$	$4.6^{\wedge}10^{12}$	$1.9^{\wedge}10^{7}$	$1.5^{\wedge}10^{3}$	$1.8^{\wedge}10^{-5}$	$7.9^{\wedge}10^{-5}$
4	$5.8^{\wedge}10^{9}$	$7.5^{\wedge}10^{8}$	$7.9^{\wedge}10^{6}$	?	-	-
5	$4.6^{\wedge}10^{10}$	$1.7^{\wedge}10^{10}$	$8.2^{\wedge}10^{9}$	?	-	-

The frequencies of the last two matings could not be calculated because no single cells were obtained on the selective medium. While there was a 'patch' visible at the non-diluted, 10fold diluted, and 100fold diluted drops, further dilution did not result in countable colonies. It may be possible that the selective media contained more tellurium than as it was indicated. However, to ensure selection and the ability to count the colonies, one needs to choose for Sistrom Km50 (without aminoacids) as selective medium for the *R. rubrum* transconjugants. However, transconjugants always need to be checked on their identity.

5.4.9 OPTIMIZED MATING PROCEDURE

5ml of the *E. coli* donor and helper strain were grown to an OD_{660} of approx. 0.8-1.5 at 37°C in LB medium. Cells (1ml) were washed twice by centrifugation with the same volume of a sterile salt solution. Chemotrophically grown cells of *R. rubrum*, grown in Sistron medium during 3-4 days, were harvested at an OD660 of approx 1-1.5, pelleted and then resuspended in the same volume of the sterile salt solution to concentrate the solution 20 times.

For triparental mating, 100µl of the washed *R. rubrum* cells was spotted on the mating plate: Sistrom supplemented with 3% peptone and 1% yeast extract. The plate was allowed to dry for 30-45 min and subsequently incubated overnight at 30°C in dark aerobic conditions. The donor *E. coli* and the helper *E. coli* were mixed with at a ratio of 1:1. The strain mixture was pelleted and resuspended in 200µl saltsolution. 50µl of the mixture spotted on top of the *R. rubrum* patches on the solid Sistrom medium enriched with peptone and yeast, resulting in a mating spot inoculated at a ratio 80:1:1 (*R. rubrum*/donor/helper). The plates were allowed to dry for 30-45 min at RT and then incubated overnight aerobically in the dark at 30°C.

In addition to the mating spot, all mating partners where separately spotted on the mating plate, whereby 100 µl of the *R. rubrum* suspension was spotted one day in advance.

The selection of the transconjugants was performed by taking the entire mating patch and resuspending it in 1ml of saltsolution. The suspension was diluted and spotted in triplicate on media selecting for the transconjugants on Sistrom medium amended with 50µg/ml Km as well as on the medium, selective for the partners. To count the mating partners, the pure patches were taken from the mating plate, resuspended in salt solution and diluted. All suspensions were tested on their own selective media (CM1962 on 869 Ni5, CM404 on 869 Km, and *R. rubrum* on Sistrom) as well as on the selective media for the transconjugants to be able to count background growth and limit of false positive conjugationfrequency.

Although Sistrom Km as a selective medium for the transconjugants provided in some cases a background of false positive colonies at a ratio of $5\text{^}10^{-8}$, other media might give an underestimation of the conjugationfrequency. However, when using Sistrom Km as selective medium, one should keep in mind that the found transconjugants need to be checked for their identity.

As negative controls a mating can be performed between CM1962 en *R. rubrum*, and CM1962 and *R. metallidurans* AE104, without the helper strain. As a positive control CM1962 can be mated with *Ralstonia metallidurans* AE104 using CM404 as a helper. The same mating procedure as described above should be used. When *R. metallidurans* AE104 is used, the selective media to count AE104 is minimal tris medium 284 with 0,2% gluconate. Transconjugants can be selected from 284 0,2% gluconate amended with Ni2mM.

5.5

5.5. Construction of a plasmidless R. rubrum

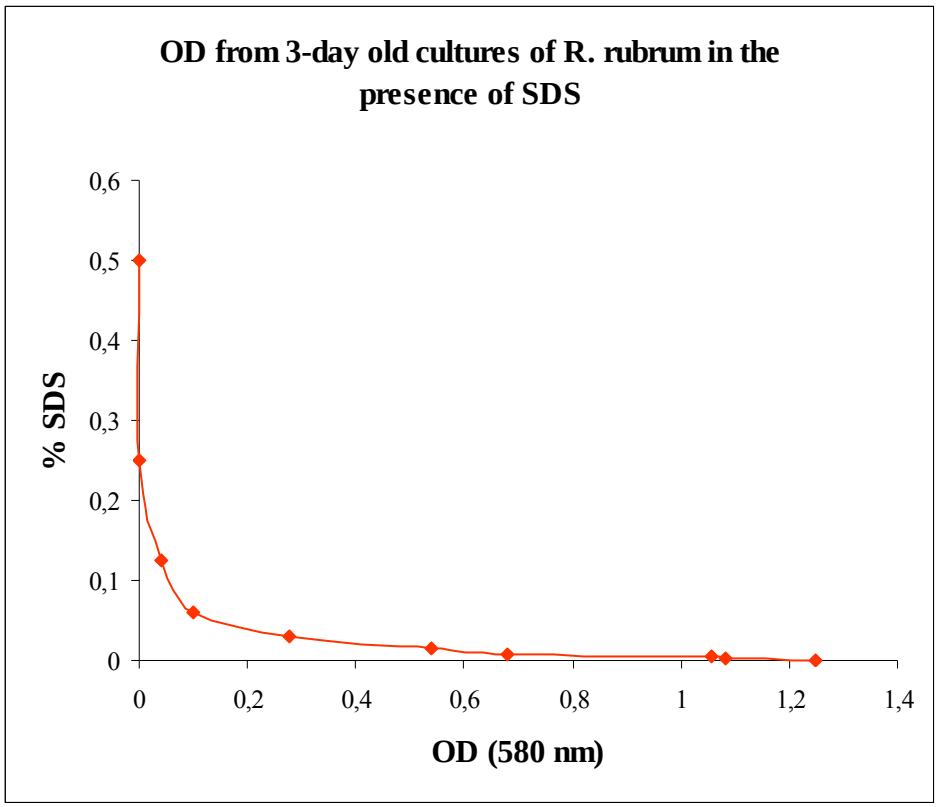

Fig. 5.3. OD of *R. rubrum* cultures grown in increased levels of SDS

Using SDS, an attempt was made to obtain a plasmid deficient *R. rubrum* strain. A plasmidless strain of *R. rubrum* would hence be usefull in mating experiments, without the fear of interference (recombination, retrotransfer, secondary transfer, ...) with the natural plasmid residing in *R. rubrum*. For that purpose *R. rubrum* was grown in the presence of SDS. The culture that was barely growing (0,125% SDS) was diluted and plated on rich medium (Sistrom peptone yeast). After 10 days of incubation, colonies lacking red pigmentation were rescued and prepared for plasmid extraction. Previous reports indicate that *R. rubrum* was deficient in pigment production after plasmid loss. No plasmid extraction was performed, because the few colonies lacking pigmentation did not give rise to positive PCR amplicons upon testing using the *R. rubrum* specific primer pair.

5.6 Conclusions

The mating protocol for *R. rubrum* was optimised for the intended mating experiments. It should be noted that transconjugants, selected on Sistrom Km, should be tested on their identity. Other selective media might underestimate real transferfrequencies.
It was impossible to cure *R. rubrum* from its plasmid. Up till now no plasmidless strain was isolated.