Technical Note TN70.3

Part A: Experimental protocols to test genetic stability in the MELiSSA loop

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Abbreviations

AFLP	amplified fragment length polymorphism
BSA	bovine serum albumine
CFDAse	Carboxyfluorescein diacetate, succinimidyl ester
CTC	5-Cyano-2,3-ditolyl tetrazolium chloride
CTF	red fluorescent formazan
DEPC	diethyl pyrocarbonate
DGGE	denaturing gradient gel electrophoresis
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTE	1,4-dithioerythritol
EDTA	ethylenediaminettraacetic acid
FDA	fluorescein diacetate
HPLC	high pressure liquid chromatography
ICM-MS	intact cell MALDI-TOFF mass spectrometry
IPTG	isopropyl-ß-D-thiogalactopyrano-side
MALDI-TOF-MS	matrix-assisted laser desorption ionization time of flight mass spectrometry
Melgen	MELiSSA Genomes
MELiSSA	micro ecological life support systems alternative
MS	mass spectrometry
NCCB	Netherland Culture Collection of Bacteria
PAGE	gel electrophoresis
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PMSF	fenylmethanesulfonylfluoride
PMT	photomultiplier
PTH	
PVDF	polyvinylidenefluoride
RNA	ribonucleic acid
RT	room temperature
SAPD	surface accessible protein detection
SDS	sodium dodecyl sulfate
SRS	sequence retrieval system
TBE	Tris-Boric acid-EDTA
TCA	trichloroacetic
UV	ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

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1. Sample processing

1.1. Proteomic methods

1.1.1. Sampling method for membrane or surface accessible proteins detection (SAPD)

1. The samples of *Arthrospira* and *Rhodospirillum* are filtrated through a glass filter system (Millipore Inc) at 4°C.
2. Resuspend the pellet immediately in the sample solution (7M urea, 2M thiourea, 0.5% (V/V) triton X-100, 2% (V/V) Pharmalyte 3-10, 65 mM DTE, and 8mM PMSF).
3. Treat the samples by sonication (2 min. at 4°C) and, finally, centrifuge at 30000 X g for 30 min at 4°C in order to remove any undissolved material.
4. Use the supernatants for 2-DE analysis.
5. The samples can be frozen in liquid N, and stored at - 80°C.

1.1.2. Sampling method for supernatant cell MALDI-TOF-MS (ICM-MS)

Differents sample preparation conditions will be used and tested.

1. Supernatants will be prepared by filtration through an 2µm filter system (Nalgene disposable filter ware: 150mL filter unit with sterile receiver) at 4°C and immediately stored at -80°C.
2. In the first, supernatant will be tested after filtration without other treatment.
3. In a second experiment, peptides and proteins will be precipitated by TCA treatment before analysis.
4. After filtration at 4 °C, the protein in the supernatant will be precipitated by TCA (trichlorooacetic acid) precipitation (10% V/V TCA, overnight at 4°C).
5. After centrifugation (15000 RPM, 1h, 4°C), the pellet will be laid directly with a Maldi-tof matrix on the sample plate.

1.2. Genomic methods

Number and volume of samples required for each time point, frequency of sampling. 3 x 10 ml of samples should be taken sterily for each of the stress conditions. One sample will serve as back-up and be stored at –20°C. The two other samples will be used as duplicates, to test the reproducibility of the results.

The sampling tubes are sterile Falcon tubes of 15 ml. The samples should be frozen at –20°C immediately or fixed with 70% ethanol, if the fresh (preferable) solution cannot be investigated.

1.3. Flow cytometry methods.

Ideally, samples should be first analysed fresh. Alternatively, if fresh analysis is not possible, a simple method for the preservation of the samples, that interfere minimally with the cellular properties of cells can be used (see preservation and storage of samples). The combined analysis of the forward and the side scatters can

allow the identification of different groups that differ in terms of size and granularity, respectively. Several aspects are critical to succesful analysis of bacterial samples :

- careful sample preservation (if necessary)
- good discrimination of populations from noise
- accurate identification of populations
- careful determination of flow sample rate

1.3.1. Preservation and storage of the samples:

Best results should be obtained on fresh samples run immediately after collection. Fresh samples can be stored at 4°C for up to 12 hours with minimal effect. Fixation will always result in cell loss (about 10%) and in change of scatters signals.

Preparation of the fixative paraformaldehyde solution requires special care. It is very difficult to completely dissolve the paraformaldehyde powder, it must be vigourously mixed in distilled water for 2 hours or more at 70°C. The solution obtained can be clarified, after cooling at room temperature, by addition of small amounts of sodium hydroxide 1N. The pH is then adjusted , but the solution must be always filtered on Whatman paper filters and then through 0.2 µm-filter before aliquoting and storage. However, after thawing, paraformaldehyde aliquots must be kept at + 4°C and should not be used beyond a week.

1.3.2. Preparation of samples to analyse DNA content from bacterial cultures

Retention of fully replicated chromosomes . To obtain bacteria that contain only fully replicated chromosomes, different substances such as rifampicin (around 150 µg/ml) or cephalexin (around 10 µg/ml) will have to be added to the culture grown to an optical density of 0.1 to 0.3. The molecular mechanism of rifampicin is an inhibition of bacterial RNA polymerase that forms a complex with rifampicin. Rifampicin prevents the ribosomes from binding to messenger RNA, and thus proteins are not produced.

Flow cytometry is useful in order to study the DNA distribution of an exponentially growing culture. When DNA of an exponentially growing *E. coli* is investigated in the absence of antibiotics, it is observed that the DNA distribution resembles that of the age distribution, there are more cells with the low DNA content corresponding to a little more than 2 fully replicated chromosomes, than cells with twice the amount of DNA. When rifampicin or cephalexin is added, the DNA occurs in discrete peaks, most of the cells have 4 fully replicated chromosomes. The number of fully replicated chromosomes corresponds to the number of origins present in the cell at the time of drug addition. Cultures will have to be preincubated for around 3 hours (time to optimise) in the presence of rifampicin and cephalexin in order to allow complete runout of replication, such that all cells contain only fully replicated chromosomes.

DNA staining:

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

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

RNAse treatment:

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

Coefficient of variation :

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

The coefficient of variation (CV; the standard deviation of a normal distribution divided by the average value) for the peaks obtained will be used for comparison of stains and concentrations of rifampicin and cephalexin.

2. Proteomic analysis of supernatant and cells

2.1. Membrane or surface accessible proteins detection (SAPD)

Flow cytometry can detect and separate specific populations of bacteria by the application of cell sorting. Using an antibody against specific surface bacterial protein would improve the detection of contaminants in a specific culture, for instance in a Melissa compartment. The proteomic approach is one of the best approaches to identify a specific surface protein for each MELiSSA bacterial culture. A proteomic approach will be used to identify specificsurface proteins for *Arthrospira* and *R. Rubrum* .

2.1.1. Determination of the protein concentration

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

1. Disolve 100mg Coomassie brillant blue G250 in 50ml 95% ethanol.
2. Add 100 ml concentrated phosphoric acid.
3. Add Water to a final volume of 200 ml.
4. Prepare samples of 100,50, 25 and 12.5ug/100ul of BSA in the same solution as your protein sample.

5. Dilute the Bradford dye concentrate 5 X with water, filter if precipitations occurs.
6. Add 5 ml of the diluted dye to each sample. The red dye will turn blue when binding to the protein, allow color to develop for at least 5 min, not longer than 30 min.
7. Read the absorbance at 595nm, prepare a linear standard curve and calculate concentration.

2.1.2. Specific labelling of bacterial surface proteins

In the first step, bacterial surface proteins have to be labelled by a specific surface probe (Sabarth, 2002).

1. Suspend bacteria in ice-cold PBS (PBS : Phosphate buffer saline), 1mM CaCl2, 0.5mM MgCl2 at an optical density at 600nm of 2.5-3.5.
2. Perform surface-labelling of the bacteria by incubation with 200uM (final concentration) sulfosuccinimidyl-6-(biotinamido)-hexanoate [s-NHS-LC-biotin (Pierce)] for 30 min on ice.
3. Stop the reaction by adding two volumes of TNKCM (50mM Tris , pH 7.4, 100mM NaCl, 27 mM KCl, 1mM CaCl2, 0.5 mM MgCl2).
4. After 10 min incubation at room temperature, the bacteria need to be sedimented by centrifugation at 3500 g for 10 min and washed three times with TNKCM.
5. Purify the biotin surface proteins by affinity chromatography on reversibly binding avidin-agarose (Boehringer).
6. After extraction, separate these proteins by two-dimensional gel electrophoresis and detect biotinylated proteins on the blots using NeutrAvidin-peroxydase staining (Pierce) and chemiluminescent visualization (ECL, Amersham).
7. Identify proteins by mass spectrometry. The specificity of these surface proteins can be further analysed by comparison with the genes included in the different genomic and proteomic databases.

2.1.3. Two- dimensional gel electrophoresis procedure

2.1.3.1. Two-dimensional gel electrophoresis:

1. Perform 2-DE in a horizontal Multiphor 2-DE set-up (Pharmacia, Uppsala, Sweden) according to Wattiez et al., 1999.
2. Rehydrate IPG strips (0.5 X 3 X 180 mm), containing Immobilines NL 3-10 and 4-7, overnight in rehydration solution (8M Urea, 0.5% (V/V) triton X-100, 2% (V/V) Pharmalyte 3-10 and 9.7 mM DTE).
3. The first dimension is then performed by applying 250 µg protein/sample in a volume of 150 µl at the anodic side.
4. Run the gels on a Pharmacia Multiphor II system equipped with a Multidrive XL power supply (Pharmacia Biotech., Uppsala, Sweden) using a 3-phase program. Set the first phase at 500V for 5h, the second phase is a linear gradient spanning from 500 V to 3500 V in 5 h, and the final phase is set at 3500 V for 14 h.
5. After electrophoresis, keep the strips at –80°C or prepare directly for the second-dimension electrophoresis (go to step 6).

6. Equilibrate the gels twice for 15 min, under gentle shaking, first in equilibration buffer (50mM Tris-HCl, pH 6.8, 6M urea, 30% glycerol, 1% (W/V) SDS) containing 20mM DTE and, next, in equilibration buffer containing 244 mM iodoacetamide.
7. Carry out second-dimension separations (SDS-PAGE) with ExcelGel XL SDS 12-14 (Pharmacia) (0.5 X 180 X 245 mm) running at 20-40 mA for about 4h. Use ExcelGel SDS buffer strips from Pharmacia.
8. Perform staining with Silver nitrate of the 2-DE gels as described by Rabilloud et al. (24). Protein pI and Mr were respectively assigned by calibration of 2D-PAGE gels with carbamylyte pI calibration markers and molecular weight standard proteins (Pharmacia Biotech, Uppsala, Sweden).These different conditions have been tested in preliminary experimentations (figure 7)

2.1.3.2. Evaluation of de 2-DE patterns

The protein patterns in the gels were analysed as digitalized images using a high resolution scanner (8 bits/pixel) in combination with PDQuest software (Bio-Rad, Richmond, CA, USA). The amount of protein in a spot was assessed as background-corrected optical density, integrated over all pixels in the spot and expressed as integrated optical density (IOD). In order to correct for differences in total staining intensity between different 2-DE images, the amounts of different spots were expressed as the percentage of the individual spot IOD per total IOD of all the spots (% IOD).

2.1.4. Identification of proteins

2.1.4.1. Electroblotting

1. Perform electroblotting of proteins onto PVDF membranes (Sequi-Blot PVDF membrane; Bio-Rad, Richmond, CA, USA) using 25mM Tris, 192 mM Glycine, 0.1 % SDS as cathode buffer and 25 mM Tris, 192 mM Glycine, 2% methanol as anode buffer.
2. Soak the membranes, before the electrotransfer, in methanol for 30s and at least 10 min in anode buffer.
3. Carry out protein transfer using a semi-dry blotting apparatus (Biolyon, France) for 1h at 24V.
4. Wash the membranes four times with MilliQ water, just before staining.
5. Visualise the PVDF membrane-bound proteins by staining with Coomassie Brillant Blue R-250.
6. Incubate the membranes overnight at RT with 250ml of 1.1% (W/V) of Coomassie Brillant Blue R-250 in methanol/ acetic acid (50%/10% (V/V)).
7. Destain the membranes with 250 ml of a methanol/ acetic acid solution(50%/10% (V/V)).
8. Dry the membranes immediately at RT and store at -4°C.

2.1.4.2. N-terminal amino acid microsequence analysis:

The spots on the PVDF membrane have to be excised and the protein N-terminal amino acid sequences can be determined at the picomole level by automated Edman Degradation of 1 pmol of protein using a Beckman LF3400D protein-peptide

microsequencer equipped with an on-line model 126 Gold system microgradient HPLC and a model 168 diode array detector (Beckman Instruments).

Carboxylmethylcysteine has to be eluted just after glutamic acid in the PTH chromatograms. All samples can be sequenced using the standard Beckman sequencer procedure 4. All sequencing reagents are from Beckman. Amino acid sequence comparisons is carried out using the FASTA or the TFASTA computer program of the Genetics Computer Group (GCG) for screening protein or nucleic acid databases. The International databases are SWISS-PROT (release 34), PIR (release 50), Genpept, Genpeptnew, GenEMBL (release 53), Genbank (release 105), GenEMBLnew (release 53.+) and IMGT (release 97.06).

2.1.4.3. Mass spectrometry:

A. ENZYMATIC DIGESTION

1. Excise spots on the gel using a sample corer (Fine Science Tools Inc.).
2. Place excised gel pieces in 1.5ml polypropylene Eppendorf tubes and wash twice in 50 ul of 50 mM NH4HCO3.
3. Destain the gel pieces, dehydrate with 50 µl of 50 mM NH4HCO3/50% acetonitrile (V/V) and dry in a centrifugal evaporator.
4. Perform enzymatic digestion with the addition of 10 µl of 0.02µg/µl trypsin (Promega Madison, WI, USA) in 25mM NH4HCO3, to each gel piece, and incubated at 37°C overnight.
5. Recover the peptide solutions and extract the gel piece twice with 10 µl of 50% acetonitrile in 5% formic acid. The combined solution needs to be concentrated in a vacuum concentrator and stored frozen until use.

B. ELECTROSPRAY IONISATION TANDEM MASS SPECTROMETRY (ESI-MS-MS)

1. Perform mass spectrometry (MS) and collision-induced dissociation (MS/MS) with a Q-TOF 2 mass spectrometer (Micromass, Manchester) equipped with a Z-spray nano flow electrospray ion (nanoESI) source and a high-pressure collision cell.
2. Dissolve samples in 50% acetonitrile in 5% formic acid, and loaded into borosilicate nanoflow tips (Protana, Denmark). For MS/MS studies, the quadrupole is used to select the parent ions, which were subsequently fragmented in a hexapole collision cell using argon as collision gas and a appropriate collision energy (typical 20-35 ev).
3. Perform data acquisition on a MassLynx system based on windows NT. MS/MS data are processed by a maximum entropy data enhancement program, MaxEnt 3TM (Micromass, Manchester). Amino acid sequences are manually deduced with the assistance of Micromass's peptide sequencing program PepSeq. The resulting sequences are searched against SWISS-PROT (release 34), PIR (release 50), Genpept, Genpeptnew, GenEMBL (release 53), Genbank (release 105), GenEMBLnew (release 53.+) and IMGT (release 97.06) and JGI *Rhodospirullum* databases using BLAST.

C. MATRIX-ASSISTED LASER DESORPTION IONIZATION/TIME-OF-FLIGHT MASS SPECTROMETRY (MALDI-TOF)

The peptides (after enzymatic digestion) were characterized using matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF : M@LDI LR, Micromass).

1. Samples are mixed (1/1, v/v) with cyano-4-hydroxycinnamic acid on the target and dried in room temperature.
2. The energy of the nitrogen laser (337 nm) used is between 1.7 to 5.5 □J. Ions are submitted to a 28 kV accelerating voltage towards the detector (4.75 kV).
3. Carry out analysis in positive polarity, without a filter, after a calibration with standard peptides in the molecular mass range 1 0086 968.7 (oxytocin, vasopressin, angiotensin I, somatostatin, insulin, hirudin).
4. Resulting fingerprint from the MALDI spectrum (usually using the top 50-100 peaks at most) can then be used to search for corresponding proteins using either the Proteinlynx Global Server of Micromass, the MS-FIT programs at the UCSF Protein Prospector site, MASCOT at Matrix Sciences, Peptide Search at Heidelberg, Profound at the Prowl site at Rockefeller, PeptIdent or MultiIdent at the Expasy site, MassSearch at the CBRG in Switzerland (Computational Biology Research Group), or MOWSE at the UK Human Genome Mapping Project Resource Centre.
5. The resulting sequences are searched against SWISS-PROT (release 34), PIR (release 50), Genpept, Genpeptnew, GenEMBL (release 53), Genbank (release 105), GenEMBLnew (release 53.+) and IMGT (release 97.06) and JGI *Rhodospirullum* databases.

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

As in the intact cell mass spectrometry technique, the supernatant, mainly its protein content, is a characteristic of one bacterial species. Until now, the protein content of the different bacterial supernatants has been characterized by two-dimensional gel electrophoresis. Mass spectrometry is also able to characterise the protein supernatant of bacterial cultures by a similar way as the ICM-MS technique. Each bacterial MELiSSA compartment, especially of the compartment III, will be characterized by a specific supernantant fingerprint. Evolution of a specific fingerprint will be also analysed. A characterisation of bacterial protein supernatant fingerprints will be done in different culture time points.

2.2.1. Mass spectrometry measurements

Mass spectrometry measurements can be performed on a M@LDI LR (Micromass, Manchester, UK) laser desorption time-of-flight instrument equipped with a nitrogen UV laser (=337 nm). The laser fluence is set just above the threshold for ion production. The mass spectrometer is used in the positive ion detection mode using an acceleration voltage of +15kV. On loading each target plate, automatic, accurate indexing of sample/reference wells is performed, followed by calibration of

the m/z range of the instrument, using the average molecular weights from a standard peptide mixture (Bradykinin, angiotensin, Glu-fibrinopeptide B, renin substrate tetraddecapeptide, ACTH (18-39) all at 1 pmol/µl, bovine insulin, 2pmol/µl and ubiquitin, 10pmol/µl). The acquisition mass range is from m/z 500 to 10000 Da. For maximum throughtput of samples the supernatant mass fingerprints are acquired automatically. Spectra from the reference wells, for lock mass calibration, are also acquired automatically. Twelve target wells are used for each organism to be entered into the database. A 1µl aliquot of matrix is applied to each target spot allowed to air-dry prior to mass spectrometry analysis. Samples are mixed (1/1, v/v) with cyano-4-hydroxycinnamic acid on the target and dried in room temperature.

2.2.2. Data analysis

Spectral data are exported from M@LDI LR as .TXT files of mass, intensity pairs and to the MicrobeLynx™ search algorithm, which challenges an appropriately selected database from a range of quality controlled supernatant bacterial reference mass spectra. The quality of the replicates is 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 is used to identify significant outliers and an average mass spectral fingerprint obtained from a minimum of ten accepted replicate spectra of an individual bacterium is 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. A comparative display of the test spectrum and the differences from the best database match is produced in a browser format.

3. rDNA-based community analysis in compartment 1

Diversity of bacterial communities has been investigated for many years using methods based on isolating and culturing. Such techniques do, however, not represent the extent of the bacterial community correctly. Recent advances in the field of molecular biology have made it possible to develop techniques which no longer require the isolation and culture of bacteria (Ranjard et al., 2000). These methods involve (i) extraction of nucleic acids and (ii) analysis of targeted sequences or the whole body of genetic information. In the following the protocols of DNA extraction, RNA extraction, PCR denaturing gradient gel electrophoresis and oligonucleotide fingerprinting of rRNA genes are put forward.

3.1. DNA extraction (Hill *et al.*, 2002)

This method describes the extraction of DNA from a complex microbial community residing in pigs feces.

3.1.1. Total DNA extraction with a modified benzylchloride extraction method

1. centrifuge 20 ml of a sample from compartment 1 at 5,000 rpm for 10 min
2. disperse 0.8g of the pellet in 5 ml of benzylchloride extraction buffer (100 mM Tris-HCl [pH 9.0], 40 mM EDTA)

3. add 100 µl of 10% sodium dodecyl sulfate and 300 µl benzyl chloride to 500 µl of the suspension
4. reserve the remaining 4.5 ml of the suspension at -20°C
5. mix the sample by vortexing
6. incubate at 50°C for 30 min, with vortexing at 5 min intervals
7. add 300 µl 3M sodium acetate (pH5)
8. mix the suspension by inversion and incubate it on ice for 15 min
9. centrifuge at maximum speed in a microcentrifuge at 4°C for 15 min to separate the aqueous and organic phases
10. transfer the supernatant to a clean tube and precipitate DNA by addition of 400 µl of isopropanol
11. centrifugate at maximum speed in a microcentrifuge for 10 min at 4°C
12. wash the pellet in cold 70% ethanol
13. dry the DNA and resuspend it in 100 µl of TE (100 mM Tris-HCl, pH 8, 1mM EDTA)

3.1.2. Total DNA extraction with a sucrose-lysozyme extraction method

1. centrifuge 20 ml of a sample from compartment 1 at 5,000 rpm for 10 min
2. disperse 0.8g of the pellet in 5ml of 25% sucrose-40 mM Tris, pH8
3. add 100 µl of lysozyme (10 mg/ml in 25 mM Tris, pH8) to 500 µl of the suspension
4. incubate the sample at 4°C for 10 min
5. add 100 µl EDTA (0.5M, pH8) and incubate again at 4°C for 10 min
6. add 1 ml of lysis buffer (62.5 mM EDTA, 50 mM Tris [pH 8], 1% [vol/vol] Triton X-100) and incubate at 4°C for 15 min with periodic mixing
7. extract the lysate twice with 25:24:1 (vol/vol/vol) phenol-chloroform-isoamyl alcohol
8. precipitate DNA by addition of 85 µl of 3M sodium acetate and 850 µl of isopropanol
9. centrifugate at maximum speed in a microcentrifuge for 10 min at 4°C
10. wash the pellet with 70% ethanol
11. dry the DNA and resuspend it in 100 µl of TE (100 mM Tris-HCl, pH 8, 1mM EDTA)

3.2. RNA extraction (Alexander and Raicht, 1998)

This extraction method describes the extraction of total RNA from human feces samples.

3.2.1. Extracting RNA from the sample

1. Place a mortar and pestle on dry ice and prechill with liquid nitrogen.
2. Add a pelleted sample from the reactor and let the liquid nitrogen evaporate.
3. Pick up a piece of 0.6 to 0.9 g sample and place it in a sterile 15 ml polypropylene tube.
4. Add 2 to 3 ml 10 mM Tris-HCl (pH 7.4), 200mM NaCl, and 1mM EDTA. Cap the tube and vortex vigorously for 1 min.

5. Pour the slurry into a sterile 1.5 ml eppendorf tube and centrifuge for 1 min at 4°C.
6. Decant the supernatant carefully.
7. Add to each of 2 or 3 1.5 ml eppendorf tubes containing 1 ml Ultraspec II reagent (Biotecx Laboratories, Houston, Texas) 0.3 ml of the stoll supernatant.
8. Shake the tubes vigorously for 30 sec and incubate on ice for 5 min.
9. Add to each tube 0.2 ml chloroform, and shake again vigurously for 30 sec.
10. Incubate on ice for 5 min.
11. Centrifuge at 12,000 g for 30 min at 4°C.
12. Rescue the upper aquaous phase from each tube carefully.Don't remove the interface, and pour it to another 1.5 ml sterile eppendorf tube.
13. Extract the solution three times with acid phenol (pH 4.5) and twice with acid phenol-chloroform (5:1).
14. Purify the RNA with RNA Tack resin by adding to each sample of 0.6 to 0.7 ml, half the volume of isopropanol (0.3-0.35 ml) and 35 µl of a slurry of RNA Tack resin (Biotecx Laboratories).
15. Vortex the tube vigorously for 30 sec and centrifuge at 12,000 g for 1 min at room temperature.
16. Remove the supernatant and wash the pellet twice with 1 ml 70% ethanol, 30% DEPCH_2O.
17. Add to each pellet 70µl DEPCH_2O.
18. Vortex the tubes vigorously for 30 sec and centrifuge at 12.000g for 1 min.
19. Carefully decant the supernatant, containing RNA, into another tube and restore at -75°C.

3.2.2. Residual removal of DNA after RNA extraction

1. Add to each 70 µl sample, containing 10-50 µg RNA, 9 µl 10×buffer [1.0 M NaCl, 0.5 M Tris HCl (pH 7.9), and 0.01 M dithiothreitol] and 1µl RNase-free DNase I (10 units/µl).
2. Incubate the samples at 37°C for 10 min.
3. Extract the sample twice with an equal volume of acid phenol-chloroform (5:1).
4. Precipitate with ethanol, resuspend in 20-50 µl DEPCH_2O, and store at -75°C.

3.3. Community analysis of PCR-Denaturing Gradient Gel Electrophoresis (Koizumi et al., 2002)

1. Perform PCR with the complement of probe EUB338 as a forward primer with GC-clamp (Amann et al., 1990) and 907r (Amann et al., 1992) as a reverse primer.
2. Perform DGGE with a D-gene or D-code system (Muyzer et al., 1993) in a gel with a denaturing gradient ranging from 20 to 60%.
3. Run samples of approximately 500 ng of PCR product and 100 ng of reamplified DNA fragment during 4 h at a constant voltage of 200 V and a temperature of 60°C.
4. Incubate the gel for 10 min in Milli-Q water containing ethidium bromide (1.0 mg/L).
5. Rinse the gel for 10 min in Milli-Q water.

6. Photograph the gel illuminated with a UV (302 nm) transilluminator system.

3.4. Oligonucleotide fingerprinting of rRNA genes (Valinsky et al., 2002)

3.4.1. rDNA library construction

1. Amplify bacterial small-subunit rDNA by PCR in 10 µl glass capillary tubes.
2. The PCR reaction mixture (100µl): 50 mM Tris (pH 8.3), 500 µg bovine serum albumin/ml, 2.5 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 250 µM, each bacterial small-subunit rDNA primer (27F[GAGCTCAGAGTTTGATCMTGGCTCAG] and 1492R[CACGYTACC TTGTTACGACTT]) at a concentration of 400 nM, 5 U of Taq DNA polymerase, and 10 µl of soil DNA composed of equal volumes of DNA from each of the replicate soil samples.
3. The cycling parameters are:

1cycle :		94°C for 2 min
35 cycles:		94°C for 5 s
		48°C for 20 s
		72°C for 40 s
1 cycle:		72°C for 2 min

4. Gel isolate PCR products and purify with a QIAquick PCR purification kit (Qiagen, Chatsworth, Calif.).
5. Ligate into pGEM-T (Promega), transforme into competent Escherichia coli JM109 (Promega), and plate on Luria-Bertani (LB) agar plates containing 100 µg of ampicillin per ml that were surface spread with 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal) and isopropyl-ß-D-thiogalactopyrano-side (IPTG).
6. For each soil treatment, pick up ~768 white colonies randomly into 384-well culture plates. Most wells contain 30 µl of LB medium supplemented with 100 µg of ampicillin per ml; the exceptions are the perimeter wells, which need to be filled with 60 µl to prevent drying.
7. For array construction (see below), shake the plates (300 rpm) overnight at 37°C in an open plastic bag (closed end of bag faces incubator fan) to limit evaporation. The next day, the plates can be stored at -70°C after 30 µl of LB medium containing 30% glycerol was added.
8. For long-term storage, grow the bacteria were overnight at 37°C without shaking in an open plastic bag, with each well containing 30 µl of LB broth supplemented with 100 µg of ampicillin per ml.

3.4.2. Array construction

1. Construct the arrays by spotting PCR-amplified rDNA onto nylon membranes.
2. The 35-µl PCR mixtures need to contain 50 mM Tris (pH 8.3), 500 µg of bovine serum albumin per ml, 2.5 mM MgCl2, each deoxynucleoside triphosphate at a concentration of 250 µM, each primer (T725 [GGCCCGACGTCGCATGCTC] and SP650 [TGGTCGACCTGCAGGCGG-C], which anneal to regions of the multiple cloning site within the vector) at a concentration of 400 nM, and 1.75 U of Taq DNA polymerase. (Note that

rDNA-specific primers were not used here because they would have also amplified E. coli rDNA from the host cell.)

3. Add the reagents to 384-well PCR plates (Marsh Bio Products, Rochester, N.Y.).
4. The add portions (0.1 µl) of freshly grown overnight cultures of the rDNA clones (described above) to the PCR reagents by using a 384-pin solid pin replicator (V & P Scientific, Inc., San Diego, Calif.). The plates were sealed with Thermo-Seal (Marsh Bio Products) by using a preheated Thermo-Sealer (ABgene, Epsom, United Kingdom) for 4 s.
5. Perform the PCR by alternately submerging the PCR plates in two water baths.
6. The cycling parameters were: 1 cycle: 94°C for 10 min
   35 cycles: 94°C for 1 min
   72°C for 2 min
   1 cycle: 72°C for 5 min
7. Spot the PCR products onto dry Hybond N+ membranes (11 by 8 cm; Amersham Pharmacia Biotech) with a surfactant-coated 0.5-µl slot pin replicator and a Multi-Print replication registration device (V & P Scientific). One microliter of each PCR product should be delivered to the membrane by two sequential spotting applications. The Multi-Print device allows the contents of four different 384-well plates to be printed onto a single 11- by 8-cm membrane, resulting in an array of 1,536 clones.

3.4.3. Array hybridisation.

1. Fix the membranes by UV cross-linking (70 mJ).
2. Immediately before hybridization, the membranes have to be denatured with 0.5 N NaOH-1.5 M NaCl (twice for 5 min on absorbent paper), neutralized with 50 mM Na phosphate (pH 7.2) (three times for 3 min on absorbent paper), covered with boiling 0.1% sodium dodecyl sulfate, allowed to cool for 15 min, and then dried for 30 min.
3. DNA oligonucleotides are end labeled with T4 polynucleotide kinase (T4 PNK) (New England Biolabs); the 4-µl reaction mixtures contained 1 µM oligonucleotide, 6 µCi of [-33P]ATP, 0.4 µl of 10x T4 PNK buffer, and 2.6 U of T4 PNK and were incubated at 37°C for 30 min.
4. Hybridization solution (1.8 ml of 5% sarcosyl-0.2 M Na phosphate [pH 7.2] containing 1 nM DNA oligonucleotide probe end labeled with 33P) has to be applied to the membranes, which were covered with plastic sheeting (thickness, 102 µm), and incubated overnight at 12°C (11).
5. Wash the arrays twice in 0.1x to 4x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 5 to 30 min at 12°C (12); (the wash conditions should be optimized empirically for each probe).
6. After washing, place the membranes briefly on absorbent paper to remove any excess fluids and then enclose it with plastic wrap to prevent drying.
7. Expose the membranes were to an Imaging Screen for 16 h and then scanned with a Personal Molecular Imager. Signal intensities with background correction can be obtained by using ImaGene 4.0 software (Biodiscovery).
8. Reuse the membranes up to five times. To remove the probe between experiments, the membranes are covered with boiling stripping buffer (1x

SSC, 0.1% sodium dodecyl sulfate, 200 mM Tris; pH 7.5), allowed to cool for 15 min, and then dried for 30 min.

4. Detection of stress related genes of the MELiSSA strains

4.1. Amplification of marker genes of stress using the degenerate PCR method

There is evidence of highly interspeciesly conserved regions or motifs of amino acids that can be used for designing degenerate primers. Degenerate primers can then be used to fish out these sequences. Sequences amplified this way can then be sequenced to confirm that the sequence is corresponding with the target gene.

Afterwards, they can be used as probes to fish out the gene of interest from a genomic library. In our case, this way of investigation is relevant because-the targeted microorganisms have genome that has not been sequenced yet. The construction of probes targetted at stress genes will thereafter enable the monitoring of stress response in each compartment of the MELiSSA loop.

4.1.1. DNA extraction

For *Arthrospira platensis*, DNA is extracted according to the protocole quoted from Vonshak (1997).

1. 500 ml of culture of A. platensis (OD600 = 1) is used for DNA extraction.
2. Filaments are harvested by centrifugation (Beckman Avanti J-30 I centrifuge) several times at 12000 rpm during 10 min at 4°C.
3. Then the pellet is washed once with sterile distilled water and centrifuged 10 min at 12000 rpm.
4. The pellet is suspended in 2 ml lysis buffer (25 per cent sucrose, 10 mM EDTA, 50 mM Tris.HCl, pH 7.5).
5. The suspension is frozen at -70°C for 30 min and thawed at 37°C.
6. Lysosyme is added to a final concentration of 1 mg.ml-1 and the suspension is incubated at 37°C for 30 min.
7. SDS (final concentration 10 mg.ml-1) and Proteinase K (final concentration 0.1 mg.ml-1) are added and the mix is incubated overnight in a water bath set at 55°C.
8. An equal volume of phenol-chloroform-isoamylalcohol (25:24:1) is added and mixed gently for 10-15 min.
9. The aqueous phase is collected by centrifugation during 10 min at 12000 rpm and RNAse treated (final concentration of DNAse free RNAse 50 µg.ml-1) at 37°C for 1h.
10. This is followed by extraction with phenol-chloroform-isoamylalcohol and chloroform-isoamylalcohol(24:1).
11. Sodium acetate is added to a final concentration of 0.3 M and the aqueous phase is gently mixed.
12. The DNA is collected by ethanol precipitation with two volumes of ice-cold ethanol.

13. A hook made by a Pasteur pipette allowes the DNA to be wound round.
14. The pellet is rinced with ice-cold 70 per cent and 100 per cent ethanol, air-dried during 30 min, dissolved in TE (10 mM Tris.HCl,pH 8, 1.0 mM EDTA) at ambient temperature and stored at -20°C.
15. If the pellet is still not dissolved, DNA is put 15 min at 65°C.

For *R. rubrum*, DNA is extracted from colonies grown on 3 ml LB medium.

1. The pellet (or a few colonies) is mixed in 50 µl of TE, with proteinase K (final concentration 100 µg.ml-1), extemporaneously prepared.
2. The suspension is incubated during 1h at 65°C, inactivated during 10 min at 95°C and centrifuged during 5 min at 10000g.
3. The supernatant is collected; centrifuged again and 40 µl of supernatant is used as DNA sample for PCR.

4.1.2. Target selection

All the stress genes selected are very important for the basal resistance metabolism of bacteria to stress. It is supposed that the proteic and nucleotidic sequences of such genes are quite well conserved among close as well as distantly related microorganisms. For *Arthrospira platensis* a closely related organism is said to be *Synechocystis* and other cyanobacteria. Close relatives of *Rhodospirillum rubrum* are *Rhodobacter sphaeroides* and *Rhodopseudomonas capsulatus*. Listed below, are the interesting target genes that have up till now already been related with stress in the literature.

THERMIC STRESS *dnaK, dnaJ, groEL, groES, clpB, clpX, rpoH, regulonB*
OXYDATIVE STRESS *oxyR, soxR, soxS, mrsA, sodA&B, katE&G&A, perA*
GENERAL STRESS *rpoS*
SOS RESPONSE *uvrA, recA, lexA*
MISMATCH REPAIR *mutS*
NITROGEN STARVATION *glnA*

To test the possibility of designing a degenerate primer, targeted at a certain gene of interest with the use of genes obtained from closely related organisms, each product of the selected stress genes (protein sequence found on the website www.expasy.org) are tested for their protein sequence homology between *Escherichia coli* (common Gram negative bacteria) and *Rhodobacter sphaeroides* and/or *R. capsulatus* (reference bacteria for *R. rubrum*). After testing with tBLASTn (http://www.doe.jgi.org) high identity percents were shown, proving that the proteins selected are good candidates to design primers. Likewise did BLAST confirm high identitical sequences (http://bioweb.pasteur.fr/seqanal/blast/) between *R. sphaeroides*

and two distant microorganisms, *E. coli* and *B. subtilis* (Gram positive bacteria). Performing a general BLAST of the proteins of *E. coli* on the NCBI website also showed that these proteins are well conserved among all the microorganism databases.

4.1.3. Primer design

Designing the primer is the most important factor in the success of the experiment, and deserves careful deliberation.

4.1.2.1. Sequence alignment

First, the amino acids sequences of proteins from distantly and closely related microorganisms need to be aligned on the "Pole Bio-Informatique Lyonnais" website (http://npsa-pbil.ibcp.fr/cgi-bin/align multilin.pl). At least two blocks of conserved amino acids should be selected to enable the design of PCR primers. Several blocks per stress protein are selected. A special attention is given to blocks containing Met (M), Trp (W) (which are coded by only one codon) or Cys(C), Asp(D), Glu(E), Phe(F), His(H), Lys(K), Asn(N), Gln(Q),Tyr(Y) (which are coded by two codons).

A further alignment is done at the nucleotide level in order to refine primer design. In order to decrease the degeneracy of the primer one can keep those nucleotides which base names change only in a very distant microorganism. Should a base be conserved thoughout the alignment then it can be expected that this particular base will also be the same in the gene sequence of the target organism.

4.1.2.2. Primer design

Tips for reducing the degeneracy of the primers:

• Judicious selection of the primer sites

The positioning of the primers is a compromise between placing them at the codons for the most conserved amino acids, and placing them at the codons for less conserved amino acids, which will imply the design of more degenerate primers.

• The use of inosine as a "neutral" base

Inosine is a purine (which occurs naturally in tRNAs) that can form base pairs with cytosine, thymidine and adenine. Recently most people have been using inosine at positions were any of the four bases might be required. Inosine can therefore be used at positions where any of the four bases might be required. Each use of inosine thus reduces the degeneracy of the primer pool 4-fold.

• Include partial codons at the ends of the primers

The various codons encoding an amino acid or a set of similar amino acids have often identical nucleotides at their first (and maybe second) positions, but different at their third position. Taking into account only the first and second nucleotide of the codon

for designing the primer, gives one or two extra positions of exact match base pairs without adding any degeneracy.

• Tolerance of primer-template mismatches

When designing degenerate primers it is sometimes prefered to allow some mismatches between primer and template rather than increasing the degeneracy of the primer. No reduction of PCR product amount is observed with primers that contain a mismatch in their middle or near, but not at, their 3' ends.

• Other considerations in primer design

Sequence:

- choose primers with GC at the 5' end
- avoid runs of 3 or more G or C at the 3' end
- avoid a T at the 3' end
- avoid mismatch at the 3' end
- avoid degeneracy in the 3 nucleotides at the 3' end
- avoid complementary sequences within a primer and between primers

Length:

-18-30 nucleotides

GC content:

- 40-60%

Tm:

- Tm = 2°C *(A+T) + 4°C * (C+G)

The primer pairs selected are then checked for their similar melting temperature (their Tm must not differ of more than 4°C and must not be above 68°C) with the Primer Express® Software v2.0 provided by Applied Biosystems. With this software, we can check also the absence of self-complementary and complementary between primer pairs.

4.1.4. PCR amplification

A number of parameters can be varied to optimize reaction conditions for degenerate primers. These include: primer concentration, magnesium concentration, template concentration, number of cycles of amplification and the temperatures and times of each step in the amplification cycle. If each of these parameters has to be varied independently. The number of possibilities quickly reaches mind boggling proportions. The choice is to fix almost all of these parameters at the standard levels that have been successful in the laboratory with classical PCR, and to vary only the one parameter that seems to be the most crucial: the temperature of the annealing step during amplification.

4.1.4.1. PCR with A. platensis DNA

Amplification reactions are performed in 50 µl of a reaction tube (Roche Diagnostic & Applied Biosystems) containing 5 units of Taq DNA Polymerase(Roche Molecular Biochemicals, Germany), PCR buffer 10X with MgCl2 included, and 0,25 mM concentration of each deoxynucleotide triphosphate and 2,5 µM of each primer (supplied by Eurogentec). The quantity of DNA was 500 ng per tube that corresponds to 5 µl of template. The choice of a high concentration of primers and DNA, even if it risks enhancing the non-specific amplifications, is decided in a first reaction in order to be sure to have PCR products.

4.1.4.2. PCR with R. rubrum DNA

Amplification reactions are performed in the same way as for *A. platensis* with the following modification: 10 µl of the supernatant collected from the protocol above is used as the DNA template. A "hot start" to 94°C is carried out during 10 min in order to prevent aspecific hybridizations from the beginning. Forty- five cycles of PCR are then performed (94°C for 1 min, X°C for 2 min, 72°C for 2 min), followed by an extension at 72°C for 10 min. All PCRs are performed on Applied Biosystems.

The annealing temperature X depends on the primer pair melting temperatures. X corresponds to the average temperature between the Tm of each primer.

Different strategies can be used to improve the PCR amplifications of the product with the expected length:

- Attempts to reduce the temperature to 35°C at the first five cycles in order to have more PCR products are performed for all primer couples.
- Temperature ramps ("Touch-down" PCR) are carried out that decrease the annealing temperature by 1°C each 2 cycles, starting from the highest estimated annealing temperature of both primers to the lowest estimated annealing temperature of both primers.
- The "Nested PCR" method was used when two pairs of primers are available, that anneal within the first PCR product, in order to synthetize more reliable nested PCR product.
- Extraction from the gel of the expected band with no contact with ethidium bromide and UV is performed with the QIAGEN kit. The fragment extracted from the gel is then used for further PCR reaction using the same set of primers.
- For each PCR amplification, negative control for DNA is included.
- PCR amplifications are improved by trial and error until the obtention of a specific band on the agarose gel appears with the expected length.

4.1.5. Visualization of the amplified products

The standard method used to separate, identify, and purify DNA fragments is electrophoresis through agarose gels. The technique is simple and rapid to perform; and capable of resolving mixtures of DNA fragments that cannnot be separated adequately by other sizing procedures. Furthermore, the location of DNA within the gel can be determined directly: bands of DNA in the gel are stained with the intercaling dye ethidium bromide.

For analyzing from 0.1-1 kb sized amplicons, a 1.5% agarose gel is made by dissolving agarose in Tris-Boric acid-EDTA(TBE) buffer and heating in a microwave oven.After cooling to about 60°C, ethidium bromide (2,7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide) is added to a final concentration of 0.1 µg.ml-1.
A gel loading buffer 6X () is applied to 10 µl or 20 µl samples electrophoresis which are run by 100 V for about an hour in the electrophoresis chamber provided by Eurogentec (Mupid-21). The size of fragments is determined by running known standards (100bp DNA Ladder, Invitrogen) with the samples, and comparing the distance the unknown fragment has migrated.

4.1.6. Sequence analysis

When the degeneration of each primer from a primers pair is below 30%, non purified PCR products is sent to Genome Express in France (Meylan 38) to be sequenced. When the degeneration is above 30%, the PCR product is cloned in the pDrive vector by T-A cloning strategy using the QIAGEN Cloning plus kit and then plasmids are isolated using the QIAPREP Spin Miniprep kit from QIAGEN. Tests of restriction analysis using EcoRI (Promega, Benelux) to check the cloning of the fragment of interest are performed in 20 µl (10 µl minipreparation, 1µl EcoRI (1u), 2µl buffer H 10X, 7 µl ddH2O) by incubation for 2 min at 37°C. The cloned PCR products are then sent to Genome Express for sequence analysis. Before sending, the PCR or cloned products are ajusted to the right concentration after quantification of the products at 260 nm with the Ultrospec 3000 UV/visible Spectrometer.

4.1. Analysis of stress induction using high density DNA arrays (Rimini et al., 2000)

4.1.1. High-density filter design

1. Libraries can be constructed by cloning size-selected genomic DNA fragments, average size, respectively, 800 and 1200 bp, in the BstXI restriction site of a M13 vector derivative, M13mp18 (New England Biolabs).
2. A total of ~2000 cloned genomic inserts need to be individually amplified by PCR, using Taq polymerase and M13 specific primers. Thirty cycles of denaturation (30 s at 94°C), annealing (60 s at 60°C) and polymerization (120 s at 72°C) can be used as PCR program.
3. Amplification success rate schould be checked by agarose-gel analysis of each individual reaction.
4. Transfer the PCR products into 12 384 well microtitre plates and gridded in duplicate onto positively charged 22 x 15 cm nylon membranes (Boehringer Mannheim) using an automated 384-pin gridding device (Genetix).
5. Denature the membranes with (0.5 M NaOH, 100 mM NaCl) for 2 min, neutralize (1 M Tris pH 7.2, 100 mM NaCl) for 2 min and UV cross-link (Stratalinker, Stratagene).
6. To mark the geometrical configuration of the array, the PCR amplified luc gene coding for the Coleoptera luciferase can be used and needs to be gridded at determined positions onto the filters.

4.1.2. RNA extraction and probe preparation

1. Isolate RNA from cell pellets with a kit (for example: the Hybaid Ribolyserrm Kit BLUE).
2. Resuspend in DEPC-treated water and measure the RNA concentration of each sample by measuring the absorbance at 260 nm, assuming 1 OD unit is equivalent to 40 wg of RNA. To check the integrity of the RNA, a small fraction of each sample will be subjected to electrophoresis on a formaldehyde agarose gel.
3. Prepare radiolabelled first strand cDNA probes using the random hexamers priming method.
4. The reaction mixture (50 wl) contains 25 wg of total RNA, 5 wg of random hexamer primers (Gibco BRL), 0.25 mM dATP, dGTP and dTTP, 2.5 wM dCTP, 50 wCi of [0,_33 P]-dCTP (3000 Ci mmol-'), 10 U of RNasin (Boehringer Mannheim), 5 mM dithiothreitol (Gibco BRL), 250 U of SuperScript II Reverse Transcriptase and 5 wl of the 5X first strand buffer (Gibco BRL).
5. Incubate the reaction at 42°C for 2 h and stop by the addition of EDTA pH 8.0 to a final concentration of 10 mM.
6. Degrate the RNA templates by incubating the mixture in NaOH 0.25 M at 68°C for 30 min.
7. After neutralization by the addition of HCl, remove unincorporated nucleotides using CentriSep spin columns according to the manufacturer's instructions (Princeton Separations).
8. The cDNA probe length profile can be assessed by electrophoresis on a 5% TBE urea polyacrylamide gel.

4.1.3. High-density filter hybridization

1. Prehybridize the high-density filters were in 10 ml DIG EasyHyb solution (Boehringer Mannheim) at 45°C for 5 h.
2. Prior to hybridization, 1/1000 volume of the luciferase probe should be added to each first strand cDNA probe and the probes after which it will be denatured for 10 min at 95°C.
3. Perform hybridization in 5 ml DIG EasyHyb, at 45'C for 3 days, with mixing on a rotisserie.
4. Wash the filters three times with a solution containing 0.1 x SSC, 0.1% SDS, at 68°C for 15 min and then expose to a phosphor storage screen for 3 days. Image data is captured on a phoshoimaging instrument.

4.1.4. Retrieval of stress induced genes

Amplify the stress induced genes with M13 specific primers. After purification of the amplified fragment the fragment is ready to be sequenced. Thereby a inventory of 800 to 1200 bp long fragments of stress relating genes will be obtained.

4.1.5. Data analysis

Process the hybridization images and compare using proprietary software. Any difference in general specific activity should be taken into account by a statistical normalization method, i.e. each spot intensity value was divided by the median of intensities on the grid. Induction or repression of a clone can be considered significant

whether the change in signal was above or below a certain predetermined fraction. Clones that meet these criteria wil progressed for further analysis.

After sequencing of the stress induced genes (see above) the identification of regulated clones can be performed by comparing the known DNA sequence of each clone with the genome sequence using BLAST (Altschul et al., 1997). The ORF or ORFs corresponding to the clone can be identified using the software packages DNASTAR (DNAStar) or CLONE MANAGER (Scientific and Educational Software). Also the closest homologue of the OFF or ORFs can be identified using BLAST against the SWISS-PROT and TrEMBL databases or TBLASTN against GenBank. In certain cases the non-redundant database at National Center for Biotechnology Information can be used to identify the closest homologue (http://www.ncbi.nml.nih.gov).

5. Polyphasic approach for monitoring genetic evolution

5.4. Genotypic analysis of microbial samples by AFLP™

As outlined in TN70.2, AFLP technology is especially suited to monitor genetic stability in microorganisms because of its high resolution and flexibility. The AFLP approach is based on the combined use of restriction enzymes and PCR to selectively amplify adaptor-tagged DNA fragments. Using a Li-Cor apparatus, amplification products (‘amplicons’) can be automatically visualised by laser excitation because one of the PCR primers is labeled with a chemical group.The end result are complex banding patterns consisting of 50-100 bands, each band representing a DNA fragment that has been selected from the bacterial chromosome in a random but controlled fashion (sequence driven).

5.4.1. Choice of restriction enzymes

The MELISSA species and the *R. metallidurans* test strain are all moderately G+C rich (51-66%; Table 3.1), and thus the combinations of *Taq*I (T/CGA) with either *Hin*dIII (A/AGCTT) or *Apa*I (GGGCC/C) were choosen. For genomes with 50-60% mol% G+C, these combinations were found to be most suitable (Janssen, 2001).

Species	mol% G+C	Genome size (Mb)
Rhodospirillum rubrum	63.8 - 65.8	3.40
Nitrosomonas europaea	51.0	2.98
Nitrobacter winogradskyi	61.2	N/A
Arthrospira platensis	53.4	~5.4
Ralstonia metallidurans	63.5	~5.81

Table 3.1: main characteristics of principle MELISSA strains

5.4.2. Design of adaptors and selective primers

In a first round of experiments, four different primer pairs will be tested for each enzyme combination. This is accomplished using the primers suggested in Table 3.2. At a later stage, depending on the first results, more combinations can be tried out by using additional Hin- or Apa-primers, i.e. H02, A02, etc. [Note: the notation of AFLP primers follows an alphanumerical code corresponding to the core sequence (A for *Apa*I, H for *Hin*dIII, T for *Taq*I) and 3'-moiety (0 for no selective base, 1 for adenine, 2 for cytosyne, 3 for guanine, 4 for thymine, 5 for two adenines, etc.)].

Enzyme	Sequence	Function	Notation	5'-3'-sequence	Length
TaqI	T/CGA	T-adaptor	TaqAd01	gACgATgAgTCCTgAC	16
			TaqAd02	CggTCAggACTCAT	14
ApaI	GGGCC/C	A-adaptor	ApaAd01	TCgTAgACTgCgTACAggCC	20
			ApaAd02	TgTACgCAgTCTAC	14
HindIII	A/AGCTT	H-adaptor	HinAd01	CTCgTAgACTgCgTACC	17
			HinAd02	AgCTggTACgCAgTC	15
		T-primers	T01	gATgAgTCCTgACCgAA	17
			T02	gATgAgTCCTgACCgAC	17
			T03	gATgAgTCCTgACCgAg	17
			T04	gATgAgTCCTgACCgAT	17
	(IRDye800)	A-primer	A01	gACTgCgTACAggCCCA	17
	(IRDye800)	H-primer	H01	gACTgCgTACCAgCTTA	17

Table 3.2: suggested AFLP primers and adapters for MELISSA strains

In order to detect DNA polymorphisms in the MELISSA strains, each genomic sample needs to be subjected to a sufficient number of AFLP reactions. The use of four different primer pairs (hence, the production of four different DNA patterns) may not be adequate in terms of genome coverage and resolution, and it is expected that more primer combinations may be required as to ascertain the presence or absence of DNA arrangements and/or nucleotide substitutions as a result of (any form of) stress.

In the future, once the genome sequence of the four MELISSA strains and the *R. metallidurans* strain are fully known (the *N. europaea* and *R. metallidurans* genome projects are in gap closure stage), virtual AFLP may be undertaken. Under virtual AFLP we understand the 'in silico' prediction of the number and precize size of expected AFLP fragments based on the entire genome sequence.

5.4.3. Preparation of template DNA

In general, procedures described by Janssen *et al.* (1996) will be followed:

1. One microgram (1 μg) of total DNA will be digested with an excess of restriction enzymes (10 Units) for 90 minutes at the appropriate temperature(s) and in a reaction volume of 10-30 μl (e.g. *Taq*I digestion is performed first at 65°C, followed by *Hin*dIII at 37°C).
2. Adaptors are added immediately after digestion to a final concentration of 0.04 μM (for *Apa*I- and *Hin*dIII-adaptors) or 0.4 μM (for *Taq*I-adaptor) and ligated to the restriction fragments in the presence of 1 Unit T4-ligase [note: adaptors are designed in such a way that the original restriction site would not be restored after adaptor-to-fragment ligation; thus, the presence of restriction enzymes during the ligation prevents fragment-to-fragment ligation].
3. DNA is precipitated, washed, and finaly dissolved in 100 μl $TE_{0.1}$ (10 mM Tris, 0.1 mM EDTA, pH 8.0).
4. Template DNAs can be stored for extended periods of time at –20°C.

5.4.4. Setting up AFLP reactions

1. A typical AFLP reaction contains 20 ng of resuspended DNA template, 6.25 ng of IRDye800 labeled primer, 30 ng unlabeled primer, 0.1 mM (each) dATP, dCTP, dGTP, dTTP, 1x PCR buffer [10 mM Tris (pH 8.4), 1.5 mM $MgCl_2$, 50 mM KCl], and 0.7 Units of DNA polymerase. The reactions are usualy set up in microgrid format (at 4°C) making use of two mixes.
2. The first mix contains distilled water, PCR buffer, and GoldStar DNA polymerase (Eurogentec).
3. The second mix contains the two primers and the four deoxynucleotides (dNTPs).
4. The reactions will be performed on a Bio-Rad i-Cycler using the following 'touch-down' profile:

   1 cycle: 60s @ 94°C
   30s @ 65°C
   60s @ 72°C

   11 cycles: 30s @ 94°C
   30s @ an annealing temperature 0.7°C lower than for each previous cycle starting at 64.3°C
   60s @ 72°C

   12-24 cycles: 30s @ 94°C
   30s @ 56°C
   60s @ 72°C

The exact number of cycles needed for optimal signal strength may be determined emperically e.g. by loading samples taken from the PCR mix at cycles 24, 30, and 36.

5.4.5. Pattern visualisation and analysis

Selectively amplified fragments will be separated through a 6.5% KB^{Plus} Gel (0.25 mm) in 1x TBE buffer (KB^{Plus}) on a Li-Cor Global IR^2 System. Separation will be done at standard conditions (1,500 V, 40 mA, 40 W, 50°C, scan speed 3). During electrophoresis, the laser/microscope scans back and forth over the width of the gel and the output current from the photodiode detectors is fed into lock-in amplifiers to discriminate the signals and eventually converted to 16-bit image data (>65,000 grayscales per pixel). On the Li-Cor system, gels are routinely run for 1.5 hours or less. The resulting image of the electrophoresis patterns are in so-called 'tagged image

file format' (TIFF) which is used to define 'tracks' for each lane of interest. This is done by importing the TIFF file into GelCompar II, a software package for image processing and normalisation (Applied Maths, Kortrijk, Belgium; www. applied-maths.com). The track resolution (Y_{res}) in the GelCompar conversion program may be reduced (i.e. to exclude the primer front). External standards are to be included every 5 lanes of each gel to achieve normalisation and allow inter- and intra-gel comparison (fig. 3.1.).

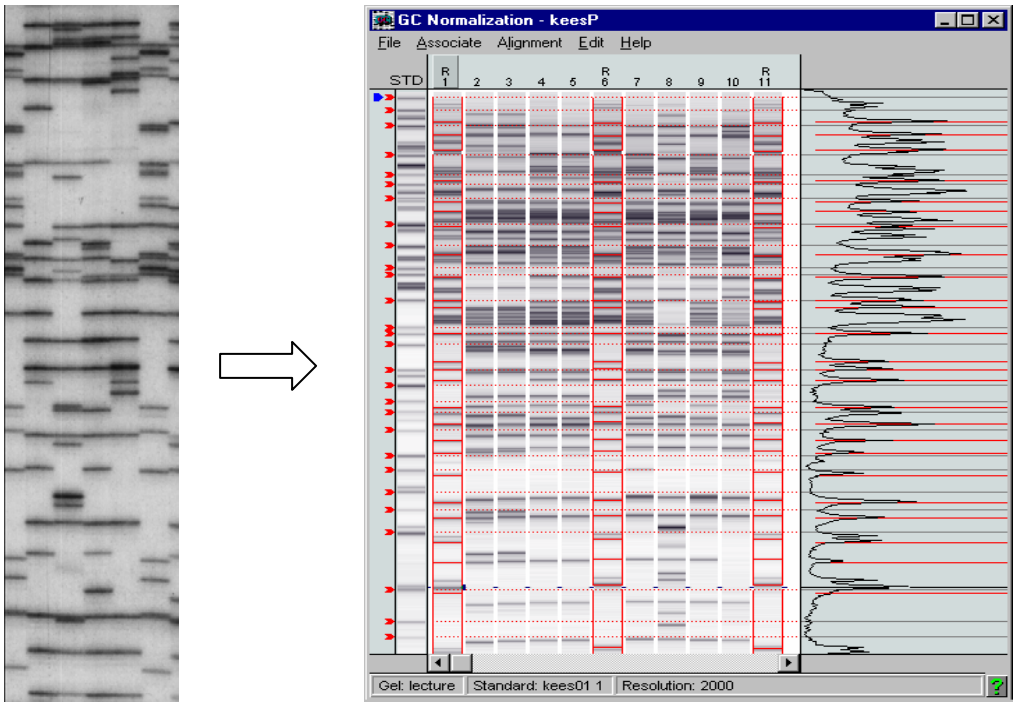


Fig. 3.1.: import of TIFF files into GelCompar (Applied Maths, Kortrijk)

5.4.6. Experimental setup

The genetic stability of the MELISSA strains will be investigated by subjecting cultures to various conditions of stress. Depending on the available facilities, this may include the exposure to controlled gamma radiation, long- and shortwave UV light, heat, cold, microgravity, and nutrient starvation (carbon, nitrogen, sulphur, and phosphorus). In addition, the possible genetic effects of long-term cultivation and the influence of MELISSA supernatans (i.e. extracellular products of another reactor strain) will be studied.

For each of the stress-condition used, cells will be harvested from the stress-exposed cultures by centrifugation (or filtration). This may be done at multiple timepoints during the exposion (to be discussed separately for each method of stress-exposure). From each sample of cells, chromosomal DNA will be prepared and purified for AFLP-based genotypic analysis. Banding patterns will be compared to the banding pattern obtained for unexposed cells and differences in the patterns will be

scored using GelCompar software. In first instance, up to four primer combinations will be used to gain a reasonable degree of genome coverage, but depending on the results, this may be extended to 8, 12, or 16 primer combinations.

Patterns that differ from the reference pattern will be further scrutinized since it is technically possible to isolate and identify polymorphic DNA by cutting out the discriminative AFLP product from either the reference or experimental run. This would involve non-automatic AFLP, isolation of the band in question from the gel matrix, re-amplification, DNA sequence analysis, and a BLAST similarity search with a local sequence database (e.g. of *R. metallidurans*) or Genbank databases. This approach may give interesting clues concerning the loci involved in or prone to stress-induced DNA re-arrangements.

6. Analysis the effect of stress through assesment of general parameters of the MELiSSA strains by Flow cytometry

6.1. Basic technical flow cytometric requirements

6.1.1. Sheath fluid

Filtered sheath is used and is preferred to distilled water because this latter can induce modifications of the refractive indexes of the cells resulting in changes of the measured forward (FSC) and side (SSC) scatters.

6.1.2. Calibration of the cytometer flow rate

To calibrate the sample flow rate, a suspension of fluorescent beads can be used. After enumeration of the beads suspension by epifluorescence microscopy, 5 to 10 replicates are analyzed by FCM under fixed delivery conditions. The actual volume (V) delivered is given by:

$$V=A/S$$

where:
A = number of beads analyzed
S = number of beads per ml determined by epifluorescence microscopy in the initial suspension or in a bead suspension with a well-known bead suspension.

Another method takes into account a precise method for measuring the calibration without the need of beads and can be applied to the EPICS XL, in which the flow rate remains relatively constant over a large period. Nevertheless, it can be affected by environmental parameters such as room temperature and must be calculated daily at the beginning and at the end of the enumeration experiments. If it is suspected that the rate varies or drifts, it must be determined every 5 or 10 samples, since its determination is critical for abundance estimates. Instability may occur in the flow rate when aggregates or big cells are present in the sample which may clog the flow cell. A pre-filtration through a 10 μm nylon mesh is necessary in such cases.

6.1.3. Protocol

1. Select a rate (Low, Medium, or High).
2. Fill a tube with the same liquid as the one containing samples (i.e., sheath Isoton ^{TM}).
3. Measure the volume of sample (or weigh precisely the tube containing the sample).
4. Place the sample in the flow cytometer and wait up to the signal "acquisition starts" appear.
5. Simultaneously, start the chronometer running.
6. Run the sample for at least 10 min.
7. Remove the sample tube and simultaneously stop the chronometer.
8. Measure (or weigh) the remaining volume.
9. Calculate the rate (R), expressed in microliters per minute, by one of the following two methods.

Volume measurement:

$$R = (V_i - V_f)/T$$

where Vi = initial volume (µl), V_f = final volume (µl), and T = time (min).

Weight measurement:

$$R = (W_i - W_f)/(T \times d)$$

where W_i = initial weight (mg), W_f= final weight (mg), T = time (min), and d = density of the liquid used for calibration (distilled water = 1.00, seawater = 1.03, for example).

6.1.4. Accuracy of measurements

Any degradation in the accuracy of measurements can be detected if standard beads are used routinely. These fluorescent beads (Immuno-check, Coulter) permit the optical alignment and parameter settings which have to be reproduced before the passing of the samples. Samples are analysed when the fluorescence of beads reach acceptable values of variation coefficient.

6.1.5. Detection and threshold.

When samples are not stained, the best approach is to start with a bacterial suspension. The acquisition is started and the needed photomultipliers (PMT 1 to PMT 4) are increased in order to obtain the main bacterial population in the middle of the screens. In all cases, it is critical to adjust the photomultiplier values in order to use all the dynamic range of the logarithmic scales. Then a flow cytometric tube containing 0.2 µm filtered sheath (Isoton^{TM}) is used. The discriminator set on the size (forward scatter) at the minimum value. The discriminator is increased until the noise can be avoided. The total number of events per second must be maintained below 100. Then the bacterial sample is run again and the discriminator adjusted again. The total number of events per second must be maintained below 1,000 per second.

6.1.6. Data acquisition.

Samples are collected as listmode and routinely 100,000 to 200,000 events are recorded typically during 2 to 10 minutes on an EPICS XL flow cytometer using the medium or high sample flow rate. Rare cells are difficult to study and require the analysis of larger sample volumes.

6.2. Optimisation of cell individualisation for *Arthrospira platensis* filaments

Arthrospira platensis grows as filaments and for most flow cytometrical applications, analyses need to be performed on single cells. Therefore, we will have to optimise the cell individualisation of *A. platensis* filaments. Different methods in order to dissociate cells will have to be performed and compared. In some cases, the cells are loosely associated and mild physical shearing techniques will release the cells from the filaments. However, this is probably not the case for *A. platensis*. Therefore, a mechanical performed (pipetting, vortex mixer, ultrasound) or chemical (enzymatic digestion) treatment might have to be. Mechanical techniques can cause significant cell damage. In some cases, enzymatic digestion is an absolute necessity. Unfortunately, most enzymes have severe effects on cells. These may be reversible or they may cause lysis. The lack of success in dissociating *A. platensis* cells from filaments may, at least in part, be due to extreme conditions necessary for dissociation of cells resulting from the age of the culture. The criteria for choice of enzyme or mechanical treatment conditions will need to be established by trial and error. After treatment, a study of the cell membrane integrity by phase contract microscopy and flow cytometry will have to be assessed.

6.3. Estimation of the abundance of the MELiSSA strains

An important parameter to take into account is the absolute (and not relative) abundance of the different cell populations. Unfortunately, most available instruments are not set to deliver well-defined sample volumes. Therefore in all cases it is necessary to precisely estimate the volume of sample analysed. On the EPICS-XL, the most accurate method consists in determining the flow rate very precisely and then recording the time of analysis for each sample.
Another possibility which is more accurate is to use beads at a known concentration (Molecular Probes) that is added to the bacterial sample. By a rule of three, it is then easily possible to calculate the concentration of the various bacterial populations.

6.3.1. Protocol

Materials:
- Bacterial samples or cultures, either fresh or frozen (see preservation and storage of the samples)
- Immunocount fluorescent microspheres (Molecular Probes) of known concentration diluted to obtain 1000 beads per ml of suspension.
- Sheath (Isoton^{TM})
- EPICS-XL flow cytometer

1. If sample has been frozen, thaw at 37°C.
2. Transfer 1 ml of sample to a suitable flow cytometer tube. If the cell suspensions are too concentrated, they can be diluted in sheath previously filtered through a 0.22-µm-pore-size filter.
3. Add 10 µl of around 10^5 beads/ml suspension of fluorescent microspheres (as an internal reference). Calibrate the flow rate of the cytometer (see Protocol of calibration of the cytometer flow rate).
4. Set the discriminator to red fluorescence and set all parameters on logarithmic amplification. Insert the sample, allow around 15 sec for the flow rate to stabilize, and then begin data acquisition. Data for natural samples are typically collected in listmode files for 2 to 4 min with a flow rate of 50 to 100 µl/min.
5. Record the time of analysis to determine precisely the cell concentrations of each population.
6. Compute the absolute cell concentration for each population in a given sample as follows:

$$\mathbf{Cpop = T \times N_{POP}/R \times (V_{total}/V_{sample})}$$

where **Cpop** = concentration of population in cells/µl, $\mathbf{N_{POP}}$ = number of cells acquired, ***T*** = acquisition time (min), ***R*** = sample flow rate (µl/min) as determined for the sample series, **V*total*** = volume (µl) of sample plus additions (fixatives, beads, etc.), and ***Vsample*** = volume of sample (µl).

Report parameters relative to the beads added to the samples:

$$\mathbf{X_{rel} = X_{pop} \times X_{beads}}$$

where $\mathbf{X_{POP}}$ is the average value of a cell parameter (scatter or fluorescence) for a given population and $\mathbf{X_{beads}}$ the same parameter for the beads.

Before calculation of the ratio, X_{pop} and X_{beads} must be expressed as linear values (not numbers of channels) after conversion from the logarithmic recording scale.

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

6.4.1. Protocol

Fresh bacteria will be passed on an EPICS XL flow cytometer at a low rate (1000 events per second). Forward and side scatters corresponding to the size and granularity will be measured and detection of variation in size and granularity monitored and compared.

6.5. Determination of genome size variation of the MELiSSA strains

In general, estimation of genome size is best assessed on fresh material on isolated nuclei. However, when a complex membrane composition prevents the release of nuclei from algae or when isolated nuclei are not stable over time, these analyses can be performed on fixed cells. Little information is available on the genome size and the base composition of the MELISSA strains.

The best way to quantify the genome size of bacteria is to use a hypotonic treatment in order to work on nuclei alone. The nuclei are released by hypotonic shock.

Depending on the species of interest, the composition of the hypotonic solution may need to be optimized. For example, addition of extra citrate gives isolated nuclei with more condensed chromatin. Increasing concentrations of detergent may help to remove membranes or cytoplasmic material attached to nuclei, which induce background fluorescence. Examination of nuclei by epifluorescence microscopy will help us to determine the best conditions.

6.5.1. Protocol

Materials:

- Hypotonic citrate solution: 50 µg/ml of propidium iodide (Sigma) in water supplemented with Tris (0.01M), $MgCl_2$ (5mM) and RNase (1 mg/ml)

Procedure:

1. To obtain bacteria that contain only fully replicated chromosomes, different substances such as rifampicin (around 150 µg/ml) or cephalexin (around 10 µg/ml) will have to be added to the culture grown to an optical density of 0.1 to 0.3.
2. Bacterial nuclei are released by hypotonic shock from 50 µl bacterial cell suspension incubated in 1 ml of hypotonic citrate solution at 37°C.
3. If isolated nuclei can not be obtained, fix the culture with 0.5 % paraformaldehyde.
4. Add 20 µl internal reference (E. Coli for example). Mix the sample by vortexing.
5. After at least 30 minutes of staining, the DNA contents of up to 15,000 cells are measured with a flow cytometer EPICS XL.
6. Measurement of DNA content is performed with both linear and logarithmic amplifications.

The measurement of DNA content for an uncharacterized or contaminating population can be obtained by comparing the mean fluorescence of this species with that of a species for which the genome size is known**.** This reference must be added before staining. If one wants to compare two strains to determine whether they have the same genome size, it is critical to work with a mixture of the strains as well as with each strain separately.

6.5.2. Use of internal reference

The DNA content is measured by comparing the mean DNA fluorescence of the species to that of a standard. The choice of the internal reference will depend on the species to be measured. If the species and the standard have genome sizes that are too different, this leads to inaccuracy in the determination of the DNA content. However, genome size is known precisely only for a small number of microbial strains that have been entirely sequenced (e.g., *Synechocystis* FTC6803 or *E. coli*).

When the genome size difference between the sample and the reference is large, one should use the data acquisition on logarithmic scale with the following formula to calculate the DNA content:

$$D = 10^{\{(F-FR) \times N/C\}} \times D_R$$

where ***D*** = DNA content of the unknown species; ***DR*** = DNA content of the reference species; ***F***= mean channel of the unknown species; ***FR*** = mean channel of the reference species; **N** = number of decades of the logarithmic amplifier; and C = number of channels used for the acquisition (256 or 1024). This formula requires precise determination of the number of decades of the amplifier used for collecting the fluorescence (Durand, 1999). It is critical not to rely on the value given by the manufacturer (2 to 4 decades), which is never accurate.

Good internal standards generally give coefficients of variation below 5%. Nevertheless, in some cases, a rapid degradation of the internal standard can be observed, due to the high hypotonicity of the isolation buffer or to some chemical compounds contained in the initial sample.

6.5.3. Isolation of nuclei

With phytoplanktonic cells, it is often difficult to isolate nuclei without damaging them (e.g. for diatoms). The method described above has been refered in order to obtain optimal results on a wide range of photosynthetic cells. However, slight modifications in the composition of the isolation buffer may be required for *Arthrospira platensis*. For example, higher detergent concentrations may help for some difficult samples. Observations with epifluorescence microscopy will help us to optimize the protocol.

7. Assessment of viability, survival, membrane permeability and membrane potential and cell cycle of MELISSA strains by flow cytometry

CAUTION : The following experiments are considered of minor importance since time and manpower will be first concentrated in order to optimise and obtain results for the above protocols (point 4.1 to 4.5) with this contract. However, we judge that some of the following protocols (point 5.1. to 5.) might be tested and compared for the MELISSA strains.

7.1. Determination of membrane permeability of the MELiSSA strains

7.1.1. Membrane permeability indicators

Propidium iodide (Sigma) and TO-PRO-1 (Molecular Probes) are both impermeant compounds that markedly increase their fluorescence on binding to double-stranded nucleic acid.

7.1.2. Membrane permeability assessment

Membrane permeability will be determined with around 100 nM TO-PRO-1 or 1 to 5 µg/ml propidium iodide (concentrations will have to be optimised). Both TO-PRO-1 and propidium iodide exhibit substantially increased fluorescences on binding to intracellular nucleic acids; both dyes normally bear positive charges and are excluded from cells with intact membranes, while they stain nucleic acids in cells with damaged membranes. Cells killed by heat exposure (conditions to be determined) will be used as controls for TO-PRO-1 and propidium iodide staining.

7.1.3. Flow cytometry measurements

Flow cytometry will be performed with an EPICS XL (Beckman Coulter). Propidium iodide and TO-PRO-1 will be excited at 488 nm and will be measured with the Photomultipliers 3 or 4. The sheath flow rate will be set at 10 µl/min and the sample analysis rate kept below 1,000 events/s.

7.2. Determination of membrane potential of the MELiSSA strains

7.2.1. Membrane potential-sensitive dyes

Diethyloxacarbocyanine iodide {$DiOC_2(3)$}; bis-(1,3-dibutylbarbituric acid) trimethine oxonol {$DiBAC_4(3)$} and Rhodamine-123 will be obtained from Molecular Probes. Rhodamine-123 will be added to a final concentration of 1 to 10 µg/ml from a stock solution of 1 mg/ml in ethanol. For $DiOC_2(3)$ and $DiBAC_4(3)$, between 1 and 30 µM final concentration will be tested.

7.2.2. Determination of membrane potential

Bacteria will be incubated with 1 to 30 µM $DiOC_2(3)$ for 4 mins, and membrane potential was estimated from the ratio of red to green fluorescence, due primarily to

emission from single dye molecules, varies with the size of the bacterial cell or clump but is largely independent of membrane potential, while the red fluorescence, due to emission from dye aggregates, is dependent on both size and membrane potential. The ratio therefore provides a cell size-independent measure of membrane potential. For comparison purposes, 1 to 30 µM $DiBAC_4(3)$ instead of $DiOC_2(3)$ will also be tested as a putative membrane potential indicator.

7.2.3. Flow cytometry measurements

Flow cytometry will be performed with an EPICS XL (Beckman Coulter). $DiOC_2(3)$ will be excited at 488 nm; its green fluorescence will be detected with the photomultiplier 1, and its red fluorescence will be detected by photomultiplier 3 or 4. The sheath flow rate will be set at 10 µl/min and the sample analysis rate is kept below 1,000 events/s.

7.3. Determination of metabolic activity (esterase activity) of the MELiSSA strains

The measurement of fluorescein diacetate (FDA) hydrolysis has been applied to estimate metabolic activity in particular esterase activity, and to help differentiating between live and dead/unhealthy cells.

7.3.1. Solution protocol

1. A stock solution of FDA (Sigma Chemicals F-7378) of 5 mg/ml will be made in dimethylsulfoxide (DMSO) and stored at 4°C.
2. The stock solution will be thawed and diluted 100-fold in distilled water; since FDA is only slightly soluble in aqueous solutions and tends to flocculate at >1 µg/ml, the stock solution will be injected fast into the ice-cold water and mixed quickly. Although the working solution might appear slightly opaque, flocculation is prevented.
3. The working solution will be kept on ice to minimize FDA degradation for max. 3 hrs and prepared fresh daily.
4. Between 20 and 500 µl FDA working solution (concentration to be optimised) will be added to 1 ml of bacterial sample (10^7 cells) in flow cytometer tubes kept at room temperature in the dark until measurement.

7.3.2. Equipment

Cells will be analyzed by an EPICS XL flow cytometer equipped with a 488 nm argon laser. Green fluorescein fluorescence will be measured on photomultiplier 1 on a logarithmic scale.

7.4. Determination of intracellular pH variations in the MELiSSA strains

Carboxyfluorescein diacetate, succinimidyl ester (5(6)-CFDAse) is the best reagent currently available in order to measure variations of intracellular pH. Viable and culturable bacteria are able to keep their pH constant when they are incubated at pH = 3 whereas non-culturable cells can not. CFDAse couples irreversibly to both

intracellular and cell-surface proteins by reaction with lysine side-chains and other available amine groups.

7.4.1. CFDAse staining protocol

Materials:

- Carboxyfluorescein diacetate, succinimidyl ester (5(6)-CFDAse); is dissolved in DMSO to 5 mmol/L, aliquoted in 50 µl and frozen in -20°C until use.
- Bovine Serum Albumin (BSA)
- Phosphate Buffered Saline (PBS)
- PBS + 1% BSA

Procedure:

1. Wash cells 3x in PBS to remove proteins.
2. Aliquot cells at a concentration of 1.10^7 cells/ml in a 15 ml tube.
3. Dilute CFDAse stock 1 : 10,000 to 0.5 µmol/L in PBS from the 5 mmol/L stock.
4. Spin cells down, remove supernatant, and add 1 ml 0.5 µMol CFDAse solution per 1.10^7 cells (concentrations between 0.1 and 5 µMol will be tested).
5. Resuspend cells well. Incubate 5 minutes at RT.
6. Wash the cells 2x with PBS / 1% BSA at 4°C to stop the labeling.
7. Cells can be used for flow cytometric assay.

NB. Due to high intensity of the signal labeling can be checked best next day.

Equipment :

Cells will be analyzed by an EPICS XL flow cytometer equipped with a 488 nm argon laser. Green fluorescein fluorescence will be assessed on photomultiplier 1 on a logarithmic scale.

7.5. Determination of respiratory activity in MELISSA .

The tetrazolium salt CTC (5-Cyano-2,3-ditolyl tetrazolium chloride) has previously been used as a viability detector in microbiology due to its ability to form intracellular granula of CTF (red fluorescent formazan) when it is reduced by an active respiratory chain.

7.5.1. Protocol

(The following protocol will be tested and optimised.)

1. Centrifuge one ml of bacteria (10^7 cells) at (8000 g, 5 min).
2. Resuspend the pellet in 1 ml sterile water at 2° C, incubated at 37° C with 5.5 mM Formate and 0.5 mM CTC for 1 h, and formaldehyde added at 2%.
3. Record CTF precipitations on an Epics XL flow cytometer.
4. Set up the flow protocol to record the precipitations by log FS, log SS, and log PMT3, with discriminator setting on PMT3 to exclude the non-fluorescent CTF negative bacteria.
5. Perform gating on logFS/logSS scattergrams, and obtain counts as PMT3 positive events.

7.6. Cell cycle analysis of MELISSA strains

Flow cytometry has been used extensively in the past to determine the cell cycle of different phytoplanktonic species in culture. In addition to providing a basic understanding of the relationships between cell cycling and environmental factors such as nutrient levels or light (Vaulot et al., 1994), the determination of the percentage of cells within the different phases of the cell cycle also provides a very elegant way to assess the division rate of the MELISSA strains. Cell cycle analysis of bacterial species is performed on fixed cells.

Flow cytometric analysis of bacteria, that have generally a very low DNA content, requires the combination of highly fluorescent stains and sensitive instruments. Nucleic acid stains are used for this purpose. However since they stain both DNA and RNA and since bacteria may have a relatively high RNA content when grown under optimal conditions, RNA must be removed enzymatically. Recently, new dyes from Molecular Probes Inc, (Eugene, Oreg.) TOTO-1, TO-PRO-1, YOYO-1, YO-PRO-1 and PicoGreen have been introduced for the detection of small amounts of nucleic acids on electrophoretic gels. TOTO-1 and YOYO-1 are cyanine dyes that are chemically different but possess similar optical properties. They present the advantages of high quantum yield and of excitation at 488 nm, a wavelength available on small flow cytometers equipped with an air-cooled laser. They emit in the green region of the spectra, are cell impermeant and can be used on fixed cells. The fluorescence of these dyes is proportional to DNA concentration and does not depend on the G-C content. The quality of DNA distributions obtained with YOYO-1 or Pico-Green on cultured samples after dilution in a low hypotonic buffer such as Tris-EDTA (10 mM Tris-HCl, 1 mM EDTA, pH 7.2), make them useful for culture studies. Very recently, it has been shown that a new dye, SYBR Green I is the most sensitive dye usable for the cell cycle analysis of bacterial cells. It has a much stronger affinity for double-stranded DNA than for RNA (Haugland, 1996; Marie et al., 1997), and is not sensitive to ionic strength.

7.6.1. Coefficient of variation

The quality of the cell cycle analysis is strongly dependent upon the coefficient of variation (CV) of the G1 peak of the linear DNA distribution. A high CV (>l0%) makes it very difficult to estimate the fraction of cells in the different phases of the cell cycle, especially the S-phase. Broad G,-like peaks result from inadequate fixation procedures or from interaction between the stain and some components of the sheath. This can also be due to a concentration of stain that is either too low or too high, leading, respectively, to nonstoichiometric or nonspecific binding. Moreover, because many bacterial species have a small genome size, the dye concentration must be lowered compared to the quantities used with mammalian or plant cells. The usual practice of filtering stock solutions through 0.2-µm-pore-size filters may induce retention of dye on the filter, thereby changing the concentration of the stock solution.

7.6.2. Protocol

1. Two ways of fixation will be tested :

1) Fix the sample by adding 500 µl of 1% paraformaldehyde (Sigma) to 500 µl of cell suspension or

2) fix the cells with cold 70%-80% ethanol while vortexing.

2. Incubate at 4°C for up to 1 hour.
3. If samples cannot be run immediately after fixation, they must be deep frozen in liquid nitrogen and stored at -80°C for delayed analysis. Frozen samples should be thawed at 37°C.
4. Add 10 µl of 1% RNase (Sigma) for every 1 ml of sample. Incubate 30 min at 37°C.
5. Add 10 µl propidium iodide, SYBR Green I, TOTO-1 or YO-PRO-1 working solution (final concentration corresponds to a 10,000-fold dilution of the commercial solution). Incubate 15 min at room temperature in the dark.

7.6.3. Cell cycle analysis

The cell cycle analysis will preferably be performed with both logarithmic and linear amplifications. The logarithmic scale is used to discriminate the populations of interest. The linear scale is required to perform cell cycle analysis. On flow cytometers such as the EPICS XL, it is possible to record both signals. Put the discriminator on the red fluorescence. Run the sample for 4 min at low rate (<50 µl/min).

Typically, samples are run at a flow rate of 30 to 50 µl/min and the number of events is kept below 1000 per sec (by diluting samples that are too concentrated). Cell cycle analysis allows one to determine the percentage of cells in the different phases of the cell cycle : G1, S, and G2 (prokaryotes) or G2/M (eukaryotes). Common problems are excessive noise and broad Gl-like peaks. Noise can result from inadequate fixative solutions. We will have to make sure that all our solutions, including buffers, dyes or detergents are free of contaminating microorganisms. However, dye stock solutions must not be sterilized by filtration through 0.2 µm because some types of membranes can adsorb dyes. Broad Gl-like peaks generally result from too high dye concentration, inducing nonspecific binding.

8. Bioinformatic approaches

8.1. Inventarisation of stress related genes

8.1.1. Stress related genes

A wide scala of various stress conditions exist for bacteria both in nature and in artificial environments (Table 8.1.).

Genes of MELISSA strains known or suspected to be involved in various stress-responses, or genes that may be up- or down-regulated as a result of stress, are to be tracked down by a literature survey and sequence data on these genes are to be gathered by bionformatic approaches.

The factors can be classified in four groups (Table 8.1): Physiological, biochemical, Biological and physical.

Other relevant genes are related to: bacterial growth, sigma factors, DNA uptake, DNA repair, SOS-response, cell differentiation, lifecycle, apoptosis (programmed cell death), cell-to-cell communication (e.g. quorum sensing), signal transduction, sporulation, survival, scavenging, adaptation, mutagenesis, and evolution

Physiological	pH * nutrient limitation (e.g. C, P, N, S starvation) * essential growth factors (e.g., vitamins) essential amino acids stationary phase events *
Biochemical	antibiotics antibacterial peptides toxic and recalcitrant compounds heavy metals xenobiotics
Biological	phages (DNA restriction / modification; lytic cycle) * microbial / fungal competitors * biofilm related stress / survival * microbial engulfment microbial predators (i.e. *Bdellovibrio*) host factors
Physical	temperature * pressure * salinity osmotic shock (physiological?) water availability (drought / dessication) * absence or excessive presence of light * oxidative stress * magnetic field ionizing radiation * UV * microgravity *

Table 8.1.: Microbial stress factors (those with immediate relevancy to the project are marked by an asterisk)

8.1.2. The Mestre website

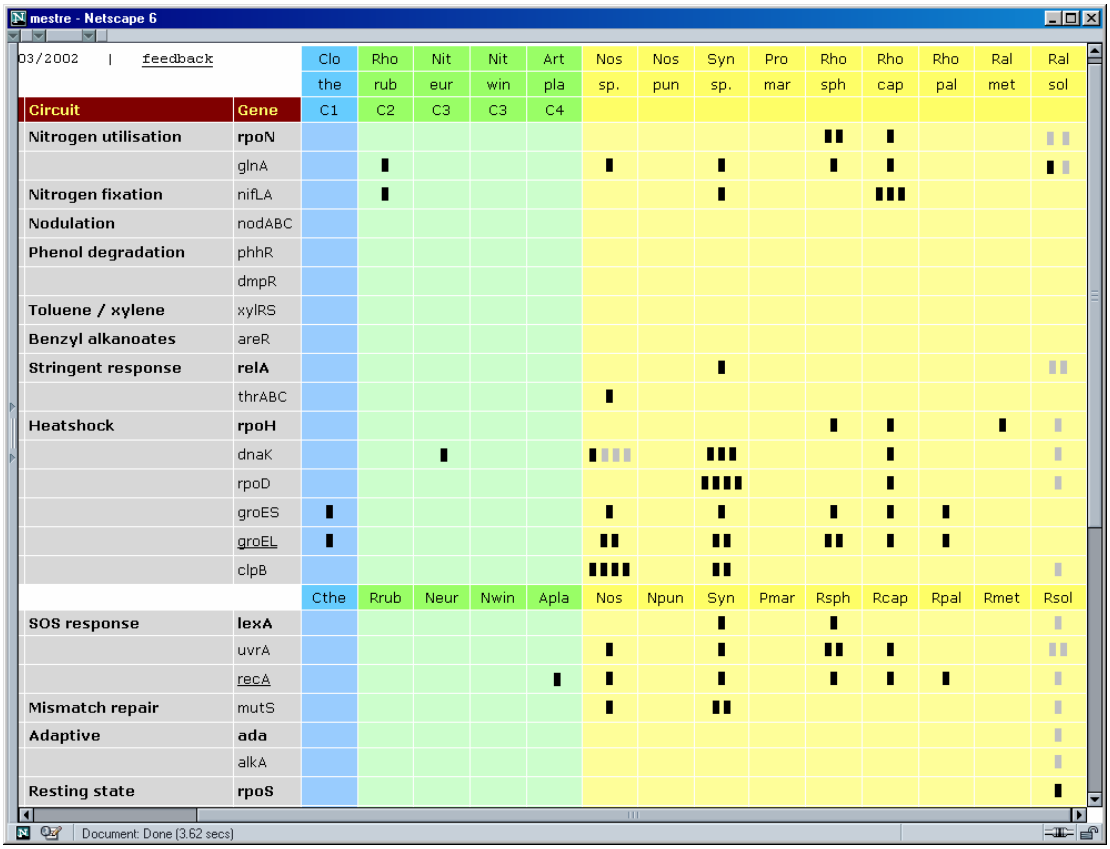
A cross-section of genes involved in bacterial stress has been presented in the Mestre website (Mestre; **Me**lissa **stre**ss-related genes) on the BSCW server (http://bscw.gmd.de/bscw/bscw.cgi/d34224338-1/*/index.htm) (Fig. 3.2.).

The table in the Mestre website was obtained by searching, for each Melissa strain separately, the Sequence Retrieval System SRS (http://srs6.ebi.ac.uk) for the following keywords in the 'gene name' search field (*):

```
  rpoN | glna | nif   | nod   | phh   | dmp   | xylR | areR | relA
| spoT | thrA | rpoH | dnaK | rpoD | groE | clpB | lexA | uvrA
| recA | mutS | ada  | alkA | rpoS | katE | bolA | oyxR | katG
| soxR | sodA | crtU
```

(*) The pipe symbol (|) in a SRS search field is used as the Boolean operator 'OR'.

The notations used in the search string are gene notations of *E. coli* or *B. subtillus* genes that are involved in various modes of stress (nitrogen starvation, oxydative response, DNA repair, etc.). An example is of this simple query is given in Fig. 3.3.



Circuit	Gene	Clo the C1	Rho rub C2	Nit eur C3	Nit win C3	Art pla C4	Nos sp.	Nos pun	Syn sp.	Pro mar	Rho sph	Rho cap	Rho pal	Ral met	Ral sol
Nitrogen utilisation	rpoN										▮▮	▮			▮▮
	glnA		▮				▮		▮		▮	▮			▮▮
Nitrogen fixation	nifLA		▮						▮			▮▮▮			
Nodulation	nodABC														
Phenol degradation	phhR														
	dmpR														
Toluene / xylene	xylRS														
Benzyl alkanoates	areR														
Stringent response	relA								▮						▮▮
	thrABC						▮								
Heatshock	rpoH										▮	▮		▮	▮
	dnaK			▮			▮▮▮		▮▮▮			▮			▮
	rpoD								▮▮▮▮			▮			▮
	groES	▮					▮		▮		▮	▮	▮		
	groEL	▮					▮▮		▮▮		▮▮	▮	▮		
	clpB						▮▮▮▮		▮▮						▮
		Cthe	Rrub	Neur	Nwin	Apla	Nos	Npun	Syn	Pmar	Rsph	Rcap	Rpal	Rmet	Rsol
SOS response	lexA								▮		▮				▮
	uvrA						▮		▮		▮▮	▮			▮▮
	recA					▮	▮		▮		▮	▮	▮		▮
Mismatch repair	mutS						▮		▮▮						▮
Adaptive	ada														▮
	alkA														▮
Resting state	rpoS														▮

Fig. 8.2.: The Mestre website – each square represent a hyperlink to sequence data obtainable from the Sequence Retrieval System (SRS)

The output of this query (fig. 8.4.) subsequently serves to introduce the corresponding hyperlinks in the Mestre website (fig. 8.2.), which are represented by clickable squares. Grey squares refer to SRS entries that contain uncertainty phrases, e.g. "DnaK-type molecular chaperone" or "Probable RNA polymerase sigma H". Each square in Mestre links to a SRS entry page on the underlying gene. For further details, please refer to the website.

Note that the Mestre website (Fig. 8.2.) lists considerable search results for organisms whose genome has been completely sequenced, but for the other strains in the table very little inormation can be obtained. It is expected that more data will come available in the next 12 months, with the completen of various genome projects.

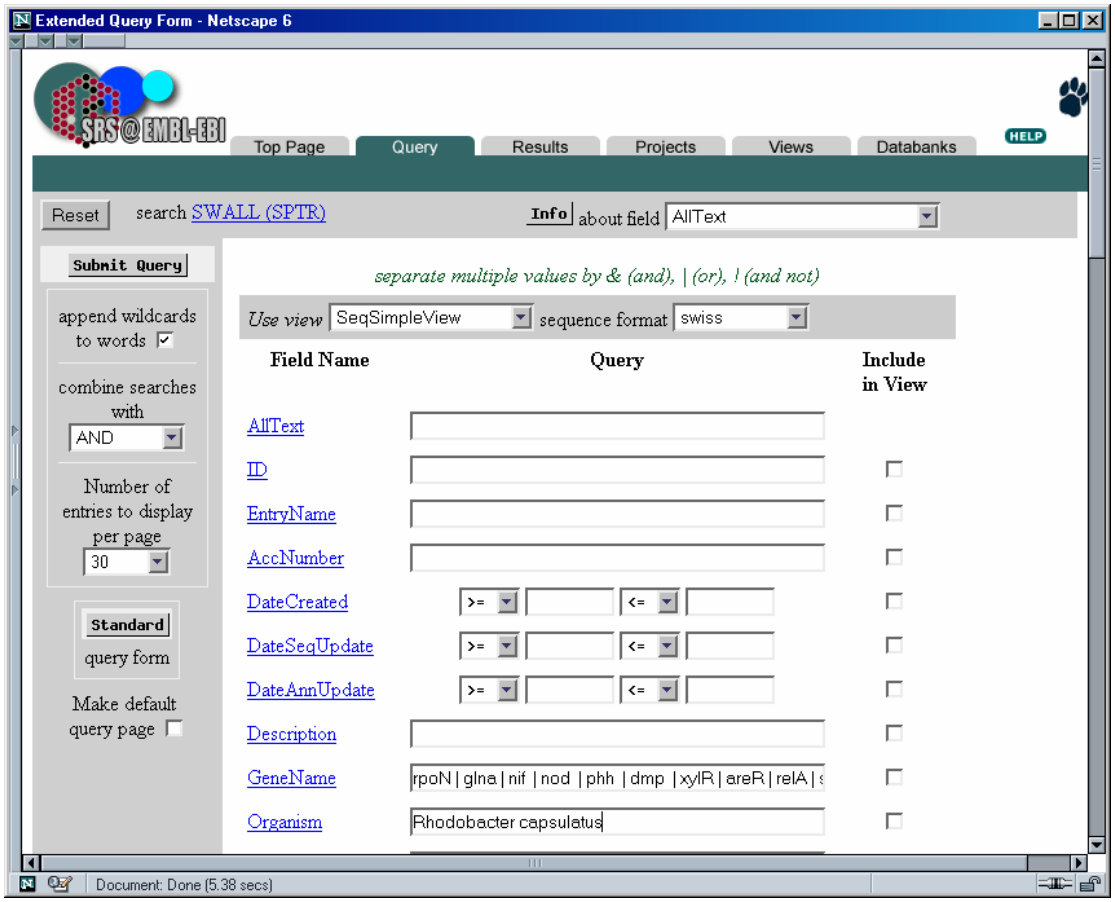


Fig. 8.3.: querying the SRS for stress-related genes

.

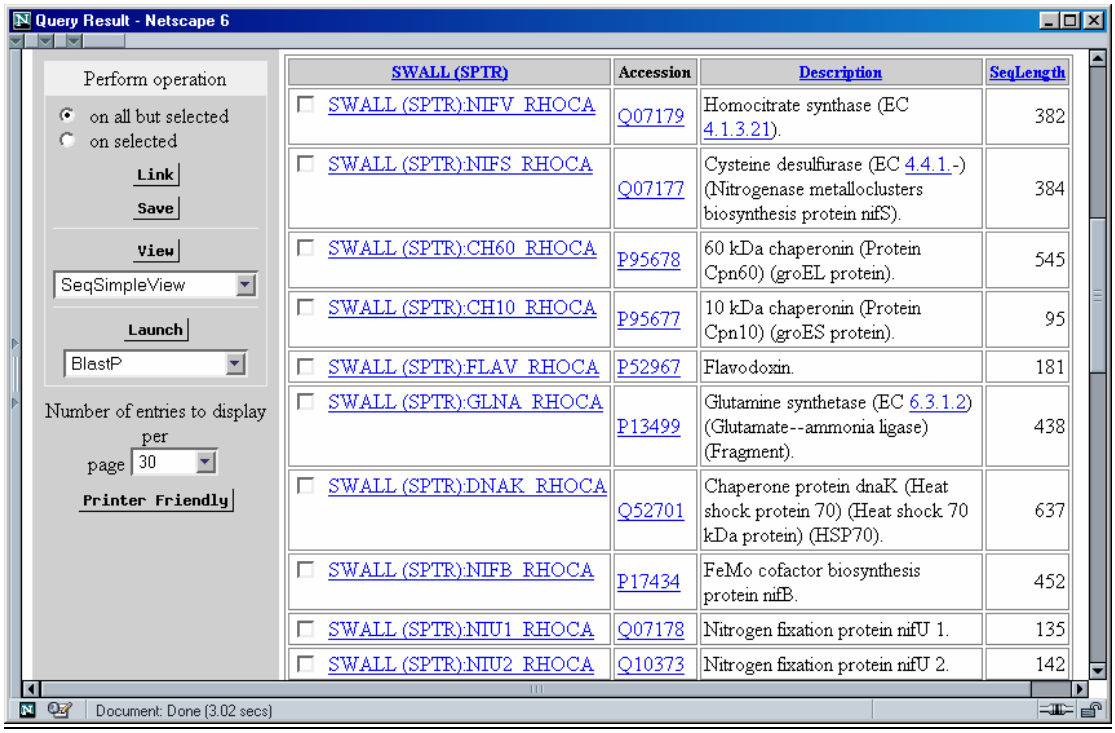
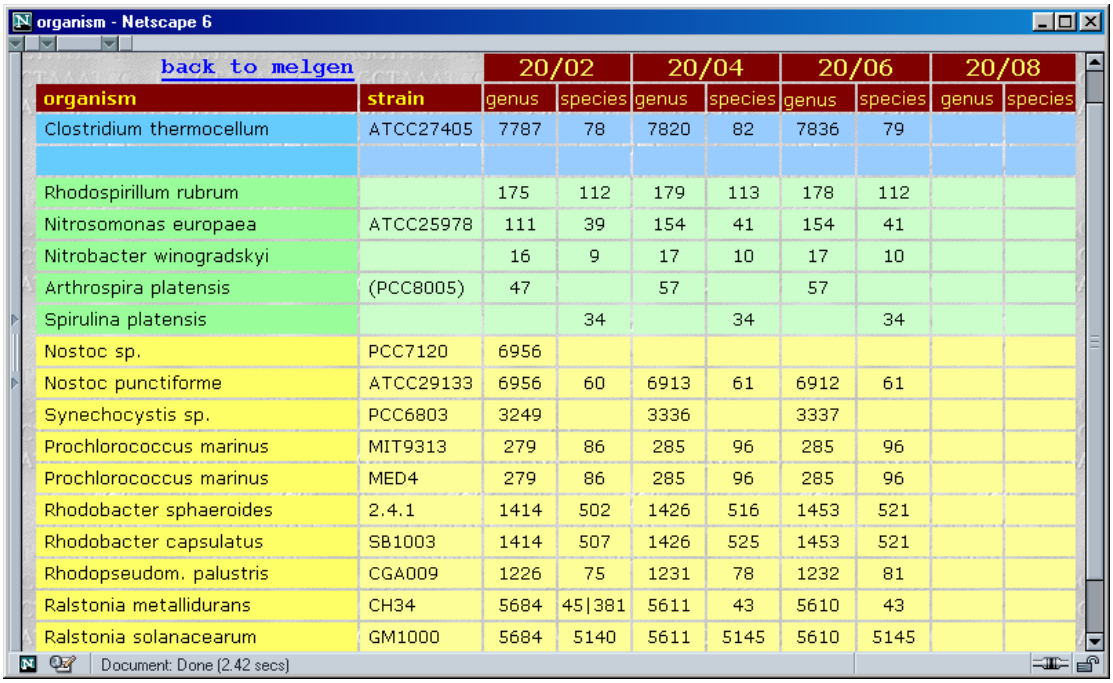


Fig. 8.4.: Typical query output produced by the Sequence Retrieval System (see text)

Ideally, a SRS-based database specific to stress genes of Melissa strains should be build. This can be done by extending the preliminary search results depicted here and in the Mestre website by using other keywords such as additional gene notations, or using the descriptions of the stress factors listed in Table 8.1. This however would entail the use of many more manhours and resources. A list of public SRS-based databases can be accessed via the SRS website (http://srs6.ebi.ac.uk). A good example is the phage database of the Netherlands Culture Collection of Bacteria (NCCB) containing 69 entries. This SRS database can be searched according to lysogenicity, collection number, plaque morphology, particle size etc. A similar approach may be undertaken for stress related genes either in general or specifically for the MELISSA project.

8.2. Permanent genomewatch

The genome watch website Melgen (**Mel**issa **gen**omes) has been discussed in detail in TN70.1. The latest version dates from 26 July 2002. An update of sequence data is executed every 2 months, but little additional data came available in a 4-month period (Fig. 8.5.). An update table can be accessed via a new menu in the Melgen website. The numbers in this table represent the number of sequences that are availbe from SRS, at genus and species level.



		20/02		20/04		20/06		20/08	
organism	strain	genus	species	genus	species	genus	species	genus	species
Clostridium thermocellum	ATCC27405	7787	78	7820	82	7836	79		
Rhodospirillum rubrum		175	112	179	113	178	112		
Nitrosomonas europaea	ATCC25978	111	39	154	41	154	41		
Nitrobacter winogradskyi		16	9	17	10	17	10		
Arthrospira platensis	(PCC8005)	47		57		57			
Spirulina platensis			34		34		34		
Nostoc sp.	PCC7120	6956							
Nostoc punctiforme	ATCC29133	6956	60	6913	61	6912	61		
Synechocystis sp.	PCC6803	3249		3336		3337			
Prochlorococcus marinus	MIT9313	279	86	285	96	285	96		
Prochlorococcus marinus	MED4	279	86	285	96	285	96		
Rhodobacter sphaeroides	2.4.1	1414	502	1426	516	1453	521		
Rhodobacter capsulatus	SB1003	1414	507	1426	525	1453	521		
Rhodopseudom. palustris	CGA009	1226	75	1231	78	1232	81		
Ralstonia metallidurans	CH34	5684	45|381	5611	43	5610	43		
Ralstonia solanacearum	GM1000	5684	5140	5611	5145	5610	5145		

Table 8.5.: Bimonthly update of the Melgen website

9. Performing stress on the MELiSSA strains

While some experiments for stress induction speak for themselves (pH, nutrient starvation, stationary phase events) others need the outline of a certain protocol (temperature, pressure, oxydative stress, water availability, absence or excessive presence of light, UV). Detection of biological stresses need first in depth analysis before being designed for each case separately. Therefore no protocol regarding biological stresses included. Two other space related stress conditions, cosmic radiation and microgravity, will likewise not be discussed because we lack the needed facilities at present to perform the experiments. Be ware that the following protocols only give a preliminary and general guide line. Conditions might have to be changed for each MELiSSA bacterium separately in order to induce a certain amount of stress optimal for obtaining maximum stress response.

9.1. Heat/Cold

9.1.1. Heat/Cold schock

1. Wash and resuspend the pellet of a culture bacterium in sterile distilled water.
2. Incubate at 42-48°C or 0-10°C during 15-120 min

9.1.2. Prolonged heat/cold

1. Grow the culture in suboptimal heat/cold conditions to mid-exponential phase in 100 ml.

9.2. Water deprivation (Agrawal and Singh, 2002)

9.2.1. Drought

1. A thick suspension of actively growing bacteria are separately placed on filter paper and kept in desiccators over fused calcium chloride at 20°C.
2. Photosynthesizing bacteria undergo a cycle of 10h light at 28µmol/m^2.s lightintensity, 20°C in dark, for 3 days.
3. Samples are resuspended in 0.01 $MgSO_4$ and prepared for analysis.

9.2.2. Physical water stress

1. A thick suspension of actively growing bacteria are inoculated on 10% agar for 15 days.
2. Photosynthesizing bacteria undergo a cycle of 10h light at 28µmol/m^2.s lightintensity, 20°C in dark, for the entire duration.
3. Samples are scratched from agar, resuspended in 0.01 $MgSO_4$ and prepared for analysis.

9.2.3. Physiological water stress

1. A thick suspension of actively growing bacteria are inoculated in liquid media containing 0.2 mol/L NaCL and kept in the culture chamber for 15 days.
2. Photosynthesizing bacteria undergo a cycle of 10h light at 28µmol/m^2.s lightintensity, 20°C in dark, for the entire duration.
3. Samples are prepared for analysis.

9.3. UV radiation

9.3.1. Photosynthesising bacteria (Agrawal and Singh, 2002)

1. A thick suspension of actively growing bacteria are placed in 10ml sterile distilled water, spread in open petri dishes and exposed to UV light from a germicidal lamp (main output at 254 nm and a fluence rate of 3.2 W/m^2). The energy fluence of UV light which was obtained by increasing the time of exposure from 30 min at 1.92 kJ/m^2.
2. The material is centrifuged, transferred to fresh media and kept in darkness for 1 d to avoid photoreactivation, whereafter the 16/8h light-dark cycle is repeated.
3. Prepare samples for analysis.

9.3.2. Non-photosynthesising bacteria

1. Expose the container, while shaking, to UV light on the UV illuminator for 6 sec.
2. Prepare samples for analysis.

9.4. Light radiation (van Waasbergen et al., 2002)

(only for photosynthesising bacteria)

9.4.1. Light intensity

1. Prior to high intensive light (HL) treatment, cultures are grown to an optical density at 750 nm (OD750) of approximately 1.0, diluted to an OD750 of 0.2 with fresh medium, and incubated for 18 h in normal light (LL) at 10 µmol photon m-2 s-1 (unless otherwise indicated).The level of HL was 800 µmol photon m-2 s-1 unless otherwise indicated.
2. Prepare samples for analysis.

9.4.2. Absence of light

1. Place an actively growing mid-exponential culture in the dark for 2 days.
2. Prepare samples for analysis.

9.5. Oxydative stress

1. Expose the photosynthesising bacteria to 50µM H_2O_2 during 1h30min. The concentration and duration of H_2O_2 exposure to *R. metallidurans* CH34 still needs to be elucidated.
2. Prepare the samples for analysis.

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Internet Resources

http://www.sb-roscoff.fr/Phyto/cyto.html
http://carl.im.uib.no/sup
http://www.flowcytometry.org
http://CCMP.bigelow.org
http://www.isac-net.org/techological/topoics/flowCytometry.htm
http://www4.umdnj.edu/cytoweb/intro.htm)

1.5. Reference list of stress induction

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