# MELiSSA

TECHNICAL NOTE

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




## TECHNICAL NOTE: 70.5

## PART A: TEST EVALUATION DOCUMENT FOR GENETIC STABILITY STUDY

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# ABREVIATIONS

| | |
|---|---|
| AFLP | Amplified Fragment Length Polymorphysm |
| BLAST | Basic Local Alignment Search Tool |
| CFDA | carboxy-fluorescein diacetate |
| CHAPS | 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate |
| CV | Coefficient of Variation |
| DGGE | Denaturing Gradient Gel Electrophoresis |
| DioC(6)3 | 3,3'-dihexyloxacarbocyanine iodide |
| DMSO | dimethylsulfoxide |
| DNA | deoxyribonucleic acid |
| dNTPs | Deoxynucleotide triphosphates |
| EDTA | Ethylene-Diamine Tetra Acetic acid |
| EtBr | Ethidium Bromide (2,7-diamino-10-ethyl-9-phenyl-phenanthridium bromide) |
| EWC | Engineering of the Waste Compartment |
| FCM | Flow Cytometry |
| FDA | fluorescein diacetate |
| HE | hydroethidine |
| HPLC | High-Pressure (or High-Performance) Liquid Chromatography |
| ICM-MS | Intact cell MALDI_TOF Mass Spectrometry |
| MALDI-TOF | Matrix Assisted Laser Desorption Ionization-Time of Flight |
| MCP | Micro-Channel Plate detector |
| MOPS | 4-Morpholinepropanesulfonic acid |
| MS | Mass Spectrometry |
| NCTC | National Collection of Type Cultures (Colindale, UK) |
| OD | Optical Density |
| OPA | One Phor All buffer |
| ORF | Open Reading Frame |
| PAGE | Pulsed Amplified Gel Electrophoresis |
| PCR | Polymerase Chain Reaction |
| PI | Propidium Iodide |
| PPM | parts per million |
| rDNA | rRNA encoding DNA |
| Rho-123 | Rhodamine-123 |
| RDP-II | Ribosomal Database Project (version 2) |
| RPM | Revolutions Per Minute |
| SAPD | Surface Accessible Proteins Detection |
| SCM-MS | Supernatant Culture MALDI-TOF MS |
| SDS | sodium dodecyl sulfate |
| TAE | Tris-Acetate-EDTA |
| TBE | Tris-Borate-EDTA |
| TCA | TrisChloroacetic Acid |
| TE | Tris EDTA |
| TEM | Transmission Electron Microscopy |
| TLF | Time Lag Focusing |
| Tris | Tris(hydroxymethyl)-aminomethane |
| UV | Ultra Violet |

# 1 INTRODUCTION

Many studies have already covered the effects of space conditions on micro-organisms. Mostly however, the results of these experiments have been inconclusive and/or contradictory. Observations as numerous and variable like increased cell density, changes in colony perimeter, growth reduction, biomass increase, increased resistance to minimum inhibitory concentrations of antibiotics, no effect on transformation or transduction, increased conjugation and increased resistance to high doses of radiation have been put forward. Nevertheless, it has been suggested that more extensive study needs to be applied to obtain reliable and conclusive evidence on the effects caused by space related environmental conditions on micro-organisms at the molecular, proteomic and genetic level.

Not only space related stress conditions will put pressure on the metabolism of the MELiSSA organisms, also the influence of processing conditions will have an effect on microbial metabolism. Heat stress caused by effluent spill of the first thermophilic compartment (run at 50°C) to the next (run at 30°C), shear stress, oxygen-, light- and nutrient-deprivation, supernatant conditions, accumulation of recalcitrant and even toxic intermediate metabolites and long-time continuous culturing will all have an influence on the health condition of the bacteria. Furthermore can the latter types of stresses even be mutagenic. It has been observed that prolonged mild temperature stress can mutagenise certain strains. Starvation conditions can likewise induce mutants to appear in single species populations.

Therefore, it is important to investigate the effect of stress on reactor stability, microbial condition and the nutritional quality of the food source. In addition it will be necessary to be able to detect stress as soon as possible for immediate intervention when this is required. Stress has already proved to be detectable in the MELiSSA organisms with a variety of methods.

In this technical note the results of the detection of stress at the cellular, the proteomic, and the genetic level.

# 2 CHANGES ASSOCIATED WITH TEMPERATURE, $H_2O_2$ AND PH STRESSES ON THE PHYSIOLOGY OF MELISSA BACTERIA USING FLOW CYTOMETRY

## *2.1. Introduction*

Not being aggregated into tissues the way plant and animal cells are, bacteria must deal with their environment individually and on intimate terms. Most animal cells are afforded the environmental luxury of being bathed in an unchanging isotonic nutrient solution maintained

at an optimal temperature and pH. Plant cells are subject to greater environemental stress, but their multicellular organization also provides them with some protection from their environment. In contrast, environmental stresses have immediate impact on a bacterial cell, so we ought not to be surprised to learn that bacteria have mechanisms to help them cope with a changing and sometimes hostile environment. Possibly because of the constant selective pressures of environmental challenge, bacteria as a group have evolved remarkable tolerance to extreme environments.

## *2.2. The effect of temperature and $H_2O_2$ on R. metallidurans*

### 2.2.1. EFFECT OF TEMPERATURE, $H_2O_2$ OR PH STRESS ON CELL SIZE AND GRANULARITY OF *R. METALLIDURANS.*

Sizes and granularities of individual bacteria were estimated cytofluorimetrically by measuring the forward and side scatters, these parameters measure light scattered at a forward and a right angle, respectively. The measurement is a complex function of size and refractive index, but for particles of bacterial size is closely correlated with volume. In principle, yeast and bacteria can be detected from the background on the basis of their intrinsic light scattering properties in forward (size) and side (granularity) scatter. The correlation between cell morphology and light scatter depends on the configuration of the optical system and can be used to estimate biomass concentration. Changes in refractive index, for example, due to the presence of inclusion bodies induced by temperature, $H_2O_2$ or pH stress can affect this relation.

Figures 1a and 2a show the effect of temperature (1a) or oxidative (2a) stress on cell size of *R. metallidurans*. No significant change (in comparison with optimal culture temperature (28°C)) could be observed in *R. metallidurans* when temperature or oxidative stress was applied.

## *2.2. Effect of temperature, $H_2O_2$ or pH stress on membrane integrity of R. metallidurans.*

The loss of membrane integrity represents significant damage for cells due to multiple functions linked to the plasma membrane (permeability barrier, transport, respiratory activity, etc...). The maintenance of membrane integrity is commonly measured in eukaryotic cells as an indicator of cell damage or cell death. All microbial cells are bounded by the cytoplasmic membrane, which allows the cell to communicate selectively with its immediate environment. Passive and active transport systems across the membrane generate an electrochemical gradient. Outside of the cytoplasmic membrane, gram-negative bacteria have an additional outer membrane. Its function is mainly protective because it prevents the entry of toxic substances into the cell. It is less selective and more permeable than the cytoplasmic membrane. An intact polarized cytoplasmic membrane and active transport systems are essential for a fully functional, healthy cell. Few fluorescent dyes used in flow cytometry can cross intact cell membranes. Most of the membrane integrity assays use nucleic acid stains (for example propidium iodide or PI in this work), due to the high concentrations of nucleic acids

within the cells and the large fluorescence enhancement exhibited by nucleic acid stains upon binding, leading to a clear separation between intact and dead cells.

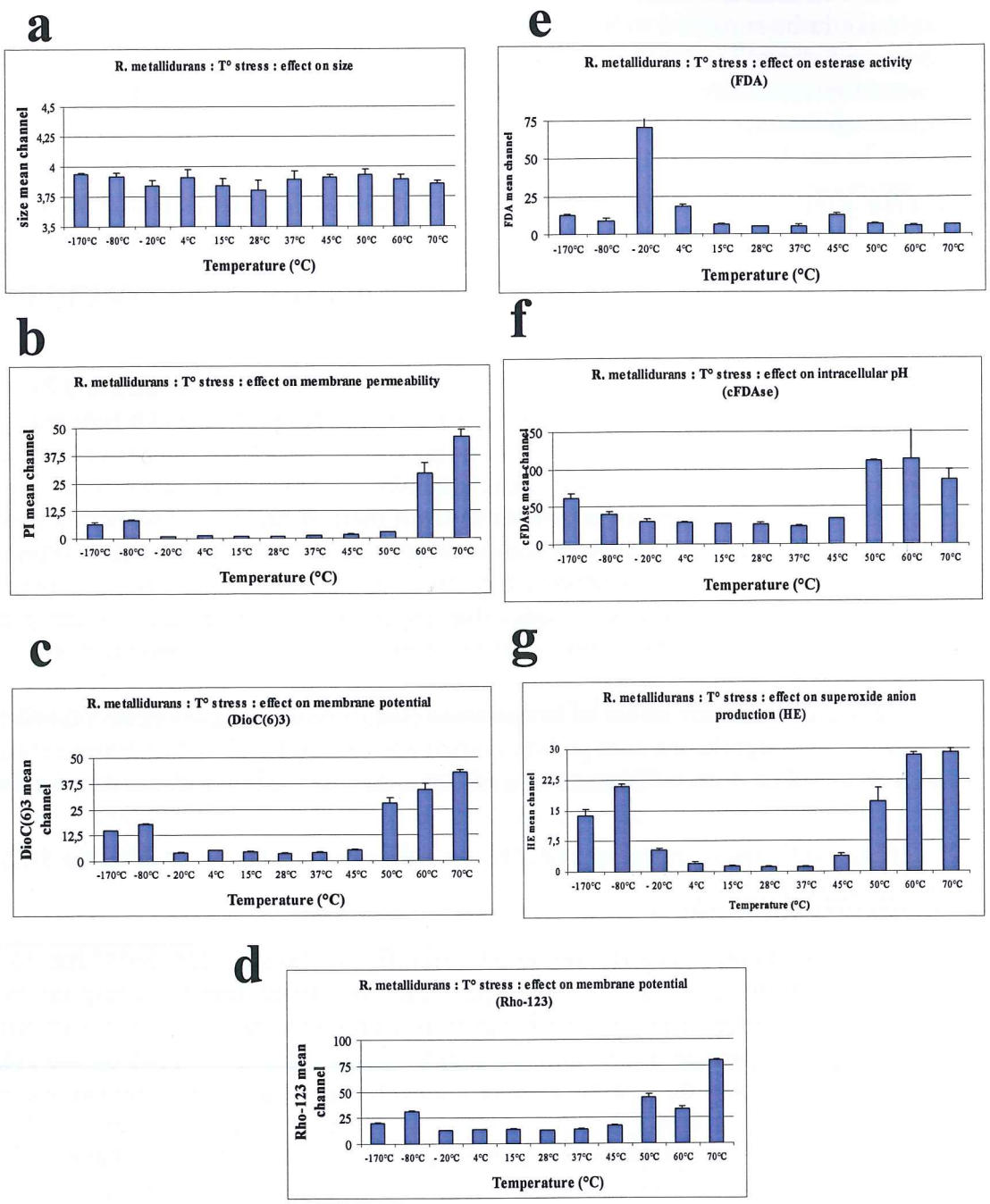



**Fig. 2.1. Effect of temperature stress on *R. metallidurans*. The effects of temperature are studied on size (a), membrane permeability (b), membrane potential (c and d), esterase activity (e), intracellular pH (f) and superoxide anion production (g).**

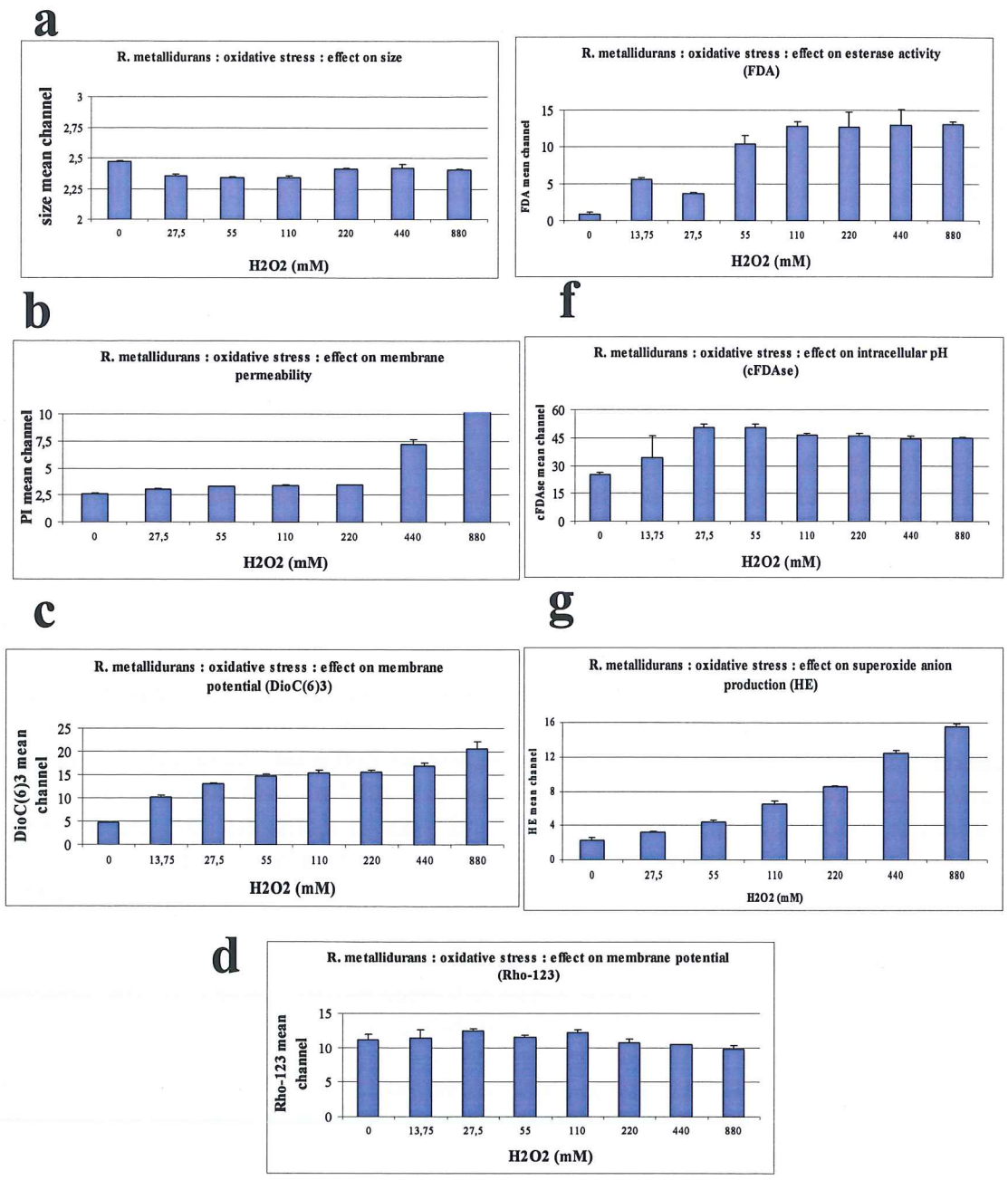



**Fig. 2.2. Effect of oxidative stress on *R. metallidurans*. The effects of $H_2O_2$ are studied on size (a), membrane permeability (b), membrane potential (c and d), esterase activity (e), intracellular pH (f) and superoxide anion production (g).**

PI is a strongly hydrophilic, small molecule ($M_r$, 660). Only cells that have lost selective permeability due to the temperature, $H_2O_2$ or pH treatment take up the dye, which stains nucleic acids. PI-positive cells fluoresce and can be quantified using flow cytometry.

Figures 1b and 2b show the effect of temperature (1b) or oxidative (2b) stress on membrane permeability of *R. metallidurans*. A significant increase of membrane permeability could be noticed when cells were submitted to low (-170°C, -80°C, -20°C) or high (+50°C, 60°C and 70°C) temperatures. Oxidative stress induced an increase in membrane permeability that was clearly proportional to the concentration of $H_2O_2$.

## 2.2.3. EFFECT OF TEMPERATURE, $H_2O_2$ OR PH STRESS ON MEMBRANE POTENTIAL OF *R. METALLIDURANS.*

The electrochemical potential occurring through the plasma membrane of metabolising bacteria is generated by respiration or by ATP hydrolysis. It results from the selective permeability of biological membranes to a variety of cations and anions leading to a difference of electric potential across the membrane. Inside, the cell is negatively charged compared with outside the cell, and membrane potential plays a central role in different cell-life processes (ATP synthesis, active transport, mobility, regulation of intracellular pH, etc.) Voltage-sensitive dyes have been developed to measure membrane potential in bacteria. Depending on the charge of the dye, they are accumulated in polarised (cationic dyes) or depolarised (anionic dyes) cells. In appropriate conditions, the amount of dye taken up can be directly related to the level of energy metabolism in the cell. DioC(6)3 and Rhodamine 123 (Rh-123) are lipophilic, cationic dyes commonly used to detect membrane potential. Accumulation inside bacterial cells is favoured by a reduction in the magnitude of the membrane potential, allowing dye molecules to concentrate within the cell, and bind to lipid-rich components. Rho-123 and DioC(6)3 have been reported to be useful to detect depolarised cells of numerous Gram-positive and Gram-negative bacterial species. DioC(6)3 is widely used as a viability stain. Bacteria with a membrane potential exclude the dye, but non-viable bacteria with depolarized membranes allow it to enter the cell. Rho-123 is a voltage sensitive cationic dye that is electrophoretically taken up into energized bacteria by virtue of the trans-membrane electrochemical potential (negative inside) of the plasma membrane.

Figures 1 cd and 2 cd show the effect of temperature (1 c and d) or oxidative (2 c and d) stress on membrane potential of *R. metallidurans*. The membrane potential of *R. metallidurans* estimated by DioC(6)3 and Rho-123 fluorescences was affected by low and high temperatures in comparison with 28°C temperature. Concerning the changes induced by oxidative stress in *R. metallidurans*, DioC(6)3 fluorescence was increased in function of $H_2O_2$ concentration whereas no significant change was observed following Rho-123.

## 2.2.4. EFFECT OF TEMPERATURE, $H_2O_2$ OR PH STRESS ON ESTERASE ACTIVITY OF *R. METALLIDURANS.*

Detection of esterase activity is measured using lipophilic, uncharged and non-fluorescent fluorogenic substrates. Once within active cells, the substrate is cleaved by non-specific esterases releasing a polar fluorescent product (fluorescein or fluorescein derivatives) retained inside cells having an intact membrane. Esterases are present in all living organisms, and these enzymes can be used to provide information on the metabolic state of bacterial cells. Although enzyme synthesis requires energy, the enzyme-substrate reaction does not, and this assay alone should be considered energy independent. However, dead or dying cells with damaged membranes rapidly leak the dye, even if they retain some residual esterase activity. Consequently, fluorogenic substrates for esterases often serve as activity and cell integrity probes that measure both enzymatic activity, which is required to activate their fluorescence, and cell-membrane integrity, which is required for intracellular retention of their fluorescent products. Fluorescein diacetate (FDA) is known to be retained efficiently inside cells with intact membrane. FDA is a nonpolar, nonfluorescent substance which enters the cells freely. Inside the cell, nonspecific esterases, among them lipase and acylase but not acetylcholinesterase, break the FDA molecule into one brightly fluorescing fluorescein and two acetates. Being highly polar, the fluorescein is trapped within cells exhibiting cell membrane integrity and the amount of fluorescence will therefore increase over time depending on the metabolic activity of those esterases. The measurement of FDA hydrolysis has been applied to estimate metabolic activity in particular esterase activity, and to help differentiating between live and dead/unhealthy cells.

Figures 1e and 2e show the effect of temperature (1e) or oxidative (2e) stress on esterase activity of *R. metallidurans*. Following temperature stress, *R. metallidurans* did not show any significant change in esterase activity except at -20°C. The oxidative stress induced on *R. metallidurans* showed that the esterase activity was increased in function of the $H_2O_2$ concentration.

## 2.2.5. EFFECT OF TEMPERATURE, $H_2O_2$ OR PH STRESS ON INTRACELLULAR PH OF *R. METALLIDURANS.*

The intracellular pH ($pH_{in}$) of bacteria is critical for the control of many cellular processes, such as DNA transcription, protein synthesis, and enzyme activities. To study regulation and maintenance of the $pH_{in}$ a reliable method to measure the $pH_{in}$ is of outmost importance. Currently, one of the most commonly used fluorescent probes for $pH_{in}$ measurements is carboxy-fluorescein diacetate, succinimidyl ester (5(6)-CFDAse) which is well suited as it is highly pH dependent and has a fast response time. 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.

Figures 1f and 2f show the effect of temperature (1f) or oxidative (2f) stress on intracellular pH of *R. metallidurans*. The temperature stress applied on *R. metallidurans* showed an increase of

intracellular pH at the freezing temperatures and also at the three highest temperatures studied. The oxidative stress induced some changes of intracellular pH as well. Indeed, in *R. metallidurans,* a slight increase of CFDAse is observed in function of $H_2O_2$ concentration.

## 2.2.6. EFFECT OF TEMPERATURE, $H_2O_2$ OR PH STRESS ON SUPEROXIDE ANION PRODUCTION OF *R. METALLIDURANS.*

Reactive oxygen species (ROS/RNS), including a series of species including superoxide anion ($O_2$-), hydrogen peroxide ($H_20_2$), hydroxyl radical, nitric oxide, and peroxynitrite, have been implicated in the etiology of cell death. $O_2$- is usually considered a precursor of more reactive species to promote the production of secondary derivatives such as hydrogen peroxide and hydroxyl radical in intracellular oxidative chain events. Thus it is a reasonable explanation that $O_2$-, as a primary signaling molecule, promotes the production of ROS downstream, which act as signaling molecules further to cause cell death through a redox sensitive pathway. Furthermore, the redox sensitive signaling may interact with more classical signaling pathways such as transcription factor activation, gene expression, and cell proliferation.

Figures 1g and 2g show the effect of temperature (1g) or oxidative (2g) stress on intracellular pH of *R. metallidurans*. *R. metallidurans* showed a U curve pattern after temperature stress meaning that the superoxide anion production was increased at the lowest as well as at the highest temperatures.

## 2.2.7. SUMMARY OF THE EFFECT OF TEMPERATURE OR $H_2O_2$ STRESS ON *R. METALLIDURANS.*

The following three tables show the statistical summary concerning the effect of temperature and $H_2O_2$ on *R. metallidurans* (one * represents a $p<0.05$ in comparison with the control (28°C in temperature experiments and 0 mM $H_2O_2$ in $H_2O_2$ experiments); two * represent $p < 0.001$). As shown in the following tables, the physiology of the cells was already slightly affected at the temperatures tested above and under the ideal growth temperature (28°C). Indeed, at 15°C and at 37°C, the cell membrane (potential and permeability) was principally altered. Above 37°C and under 4°C, *R. metallidurans* appears to be damaged at the level of its membrane (as shown by the changes in membrane permeability and potential). Within the cytoplasm, enzymes, intracellular pH and ROS production are also affected to a level that could be explained by an induction of cell death at those temperatures. With regard to the effect of oxidative stress on *R. metallidurans*, cells showed sensitivity to $H_2O_2$ concentrations from 13.25 mM meaning that an oxidative stress severely damages the physiology of *R. metallidurans*.

| *T° (°C)* | *-170°C* | *-80°C* | *-20°C* | *4°C* | *15°C* | *37°C* | *45°C* | *50°C* | *60°C* | *70°C* |
|---|---|---|---|---|---|---|---|---|---|---|
| *Size* | | | | | | | | | | |
| *Mbr. Perm.* | * | * | | ** | * | ** | * | * | * | * |
| *Mbr. Pot. (Dioc)* | ** | ** | | * | * | | * | * | * | ** |
| *Rho-123* | ** | ** | | | | | * | ** | ** | ** |
| *Esterase* | * | * | * | ** | | | * | | | * |
| *Activity* | | | | | | | | | | |
| *Intracellular pH* | * | * | | | | | * | ** | ** | * |
| *Superoxide* | * | ** | ** | * | | | * | * | ** | ** |
| *anion production* | | | | | | | | | | |

| $H_2O_2$ (mM) | 13,25 | 27,5 | 55 | 110 | 220 | 440 | 880 |
|---|---|---|---|---|---|---|---|
| Size | ** | ** | ** | ** | ** | | ** |
| Mbr. Perm. | * | ** | * | ** | ** | ** | ** |
| Mbr. Pot. (Dioc) | ** | ** | ** | ** | ** | ** | ** |
| Rho-123 | | | | | | | |
| Esterase | ** | ** | ** | ** | * | * | ** |
| Activity | | | | | | | |
| Intracellular pH | | ** | ** | ** | ** | ** | ** |
| Superoxide | * | * | ** | ** | ** | ** | ** |
| anion production | | | | | | | |

## 2.3. The effect of temperature, $H_2O_2$ and pH stresses on the physiology of R. rubrum ATCC25903

### 2.3.1. EFFECT OF TEMPERATURE, H2O2 OR PH STRESS ON CELL SIZE AND GRANULARITY OF *R. RUBRUM.*

Figure 2.3. shows representative dot-plots (side versus forward scatters) of *R. rubrum* following temperature, $H_2O_2$ or pH stress (1st, 2nd or 3rd row, respectively) whilst figure 7a shows the effect of temperature, $H_2O_2$ and pH stress on cell size of *R. rubrum*. Shifts towards lower size and granularity could be observed after incubation at 45°C, 50°C or 60°C whilst a significant diminution of the size and granularity could be noticed with all $H_2O_2$ concentrations tested. Concerning pH treatment, extreme pH induced a diminution in cell size and granularity.

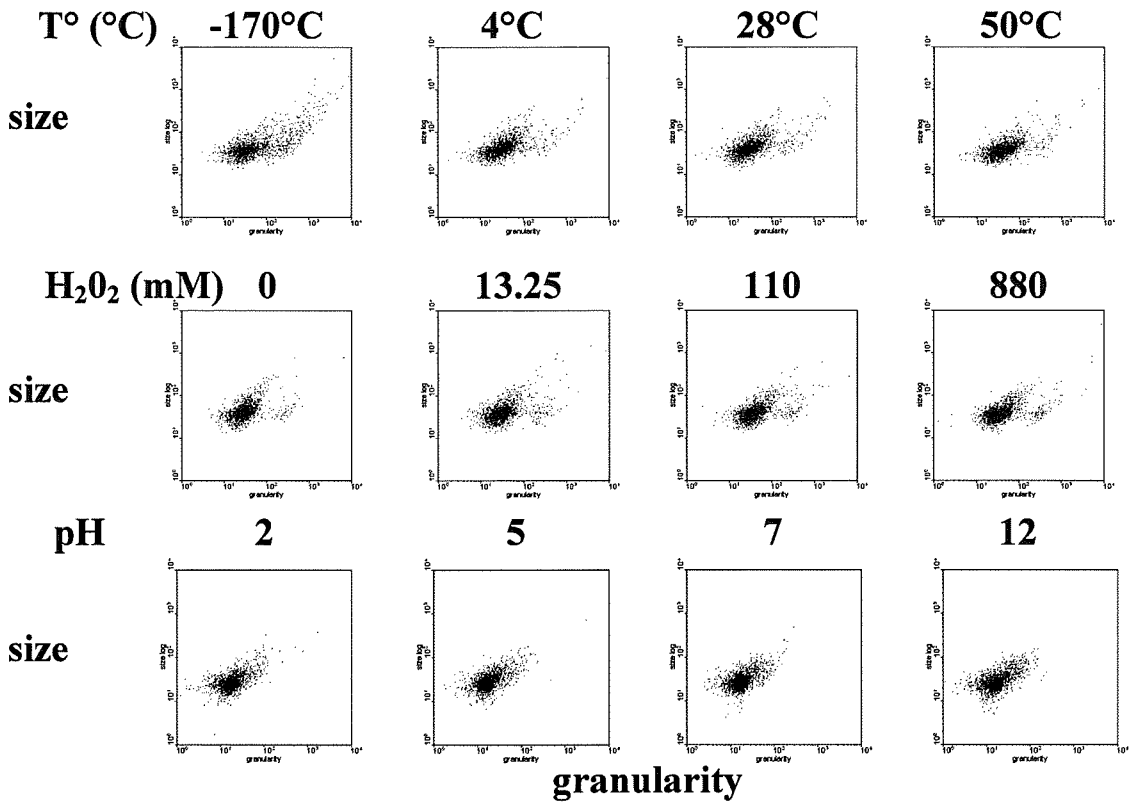



**Fig. 2.3. Representative dot-plots (side versus forward scatters) of *R. rubrum* after a 1 hour incubation at either -170°C, 4°C, 28°C or 50°C, in the presence of 13.25, 110 or 880 mM $H_2O_2$ or at pH 2, 5, 7 or 12.**

## 2.3.2. EFFECT OF TEMPERATURE, $H_2O_2$ OR PH STRESS ON MEMBRANE INTEGRITY OF *R. RUBRUM.*

Figures 4, 5 and 6 (1st row) show representative histograms of PI fluorescence in *R. rubrum* in stress conditions and figure 7b shows the effect of temperature, $H_2O_2$ and pH stress on membrane permeability of *R. rubrum.* A significant increase of membrane permeability could be measured when cells were submitted to low (-170°C, -80°C, 20°C) as well as high (45°C, 50°C, 60°C and 70°C) temperatures. Oxidative stress induced an increase in membrane permeability that was clearly proportional to the concentration of $H_2O_2$ in *R. rubrum.* pH stress only induced a significant increase in membrane permeability at pH 2 and 12.

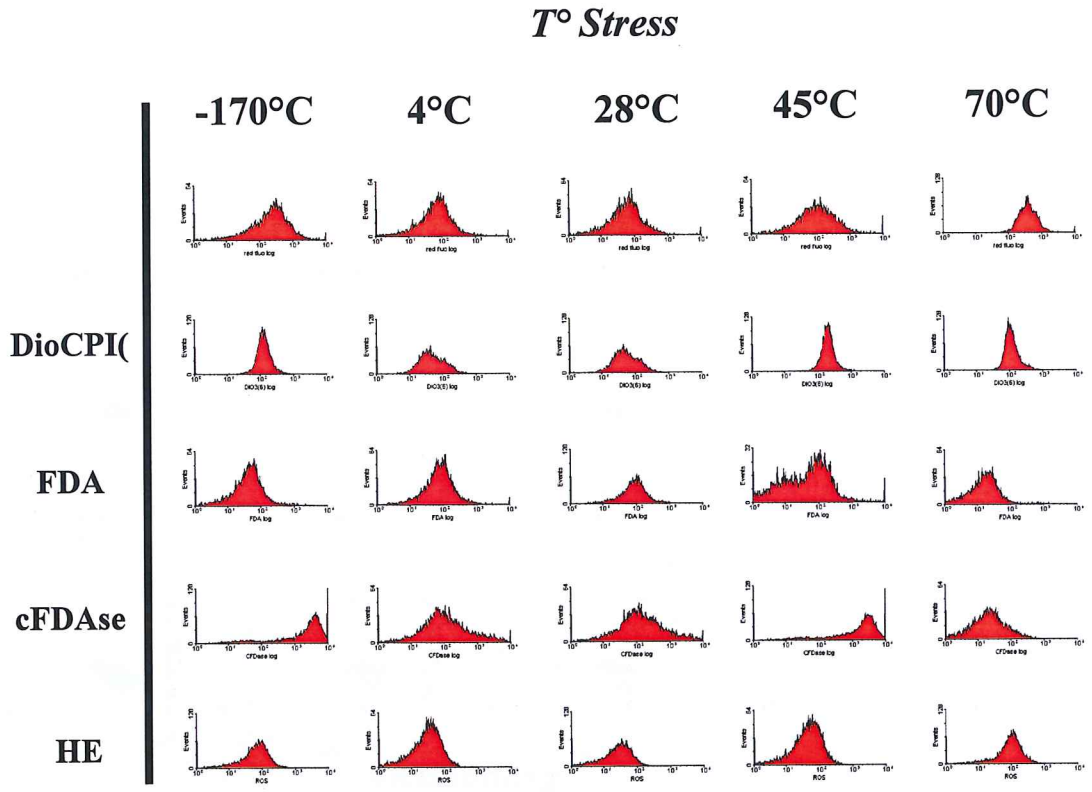



**Fig. 2.4. Representative histograms of PI (1st row), Dioc(6)3 (2nd row), FDA (3rd row), cFDase (4th row) and HE (5th row) fluorescences in *R. rubrum* following temperature stress at -170, 4, 45 or 70°C. A temperature of 28°C is considered as control.**

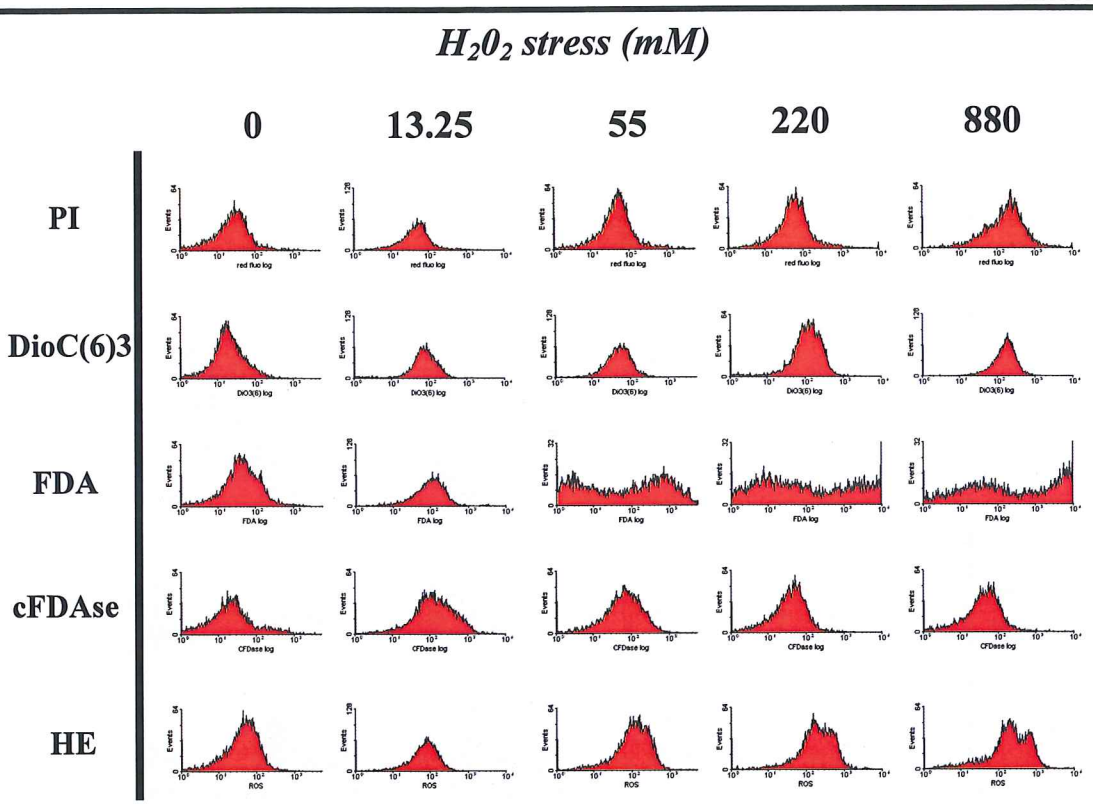



**Fig. 2.5. Representative histograms of PI (1st row), Dioc(6)3 (2nd row), FDA (3rd row), cFDase (4th row) and HE (5th row) fluorescences in *R. rubrum* following oxidative stress at 13.25, 55, 220 or 880 mM $H_2O_2$.**

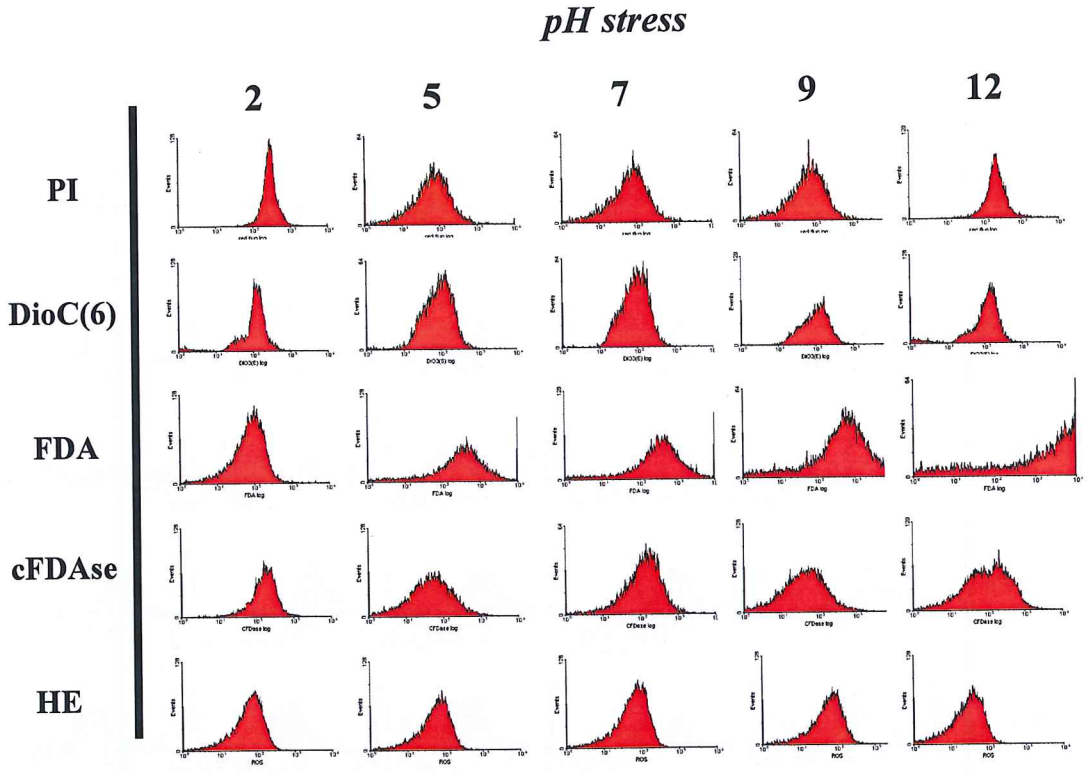



**Fig. 2.6. Representative histograms of PI (1st row), Dioc(6)3 (2nd row), FDA (3rd row), cFDase (4th row) and HE (5th row) fluorescences in *R. rubrum* following pH stress at pH 2, 5, 9 or 12. pH 7 is considered as control.**

## 2.3.3. EFFECT OF TEMPERATURE, $H_2O_2$ OR PH STRESS ON MEMBRANE POTENTIAL OF *R. RUBRUM*

Figures 4, 5 and 6 (2nd row) show representative histograms of DioC(6)3 fluorescences in various stress conditions. Figures 7c and 8a show the effect of temperature, $H_20_2$ or pH stress on membrane potential after DioC(6)3 and Rho-123, respectively of *R. rubrum* in all the stress conditions tested. The variation of membrane potential of *R. rubrum* estimated by DioC(6)3 and Rho-123 fluorescences did not give the same results. Whilst DioC(6)3 fluorescence was significantly decreased at -20°C, 4°C, 15°C and 37°C, it increased at -170°C, -80°C and from 45°C onwards. However, Rho-123 staining only revealed a change in the staining at -80°C. Concerning the changes induced by oxidative stress in *R. rubrum*, DioC(6)3 fluorescence was increased in function of $H_2O_2$ concentration from 27.5 mM whereas no significant change was observed following Rho-123. Finally, pH stress showed an increase of DioC(6)3 staining at pH 10 and 12 whereas with Rho-123, pH 2 was the only pH showing a significant difference in comparison with pH 7.

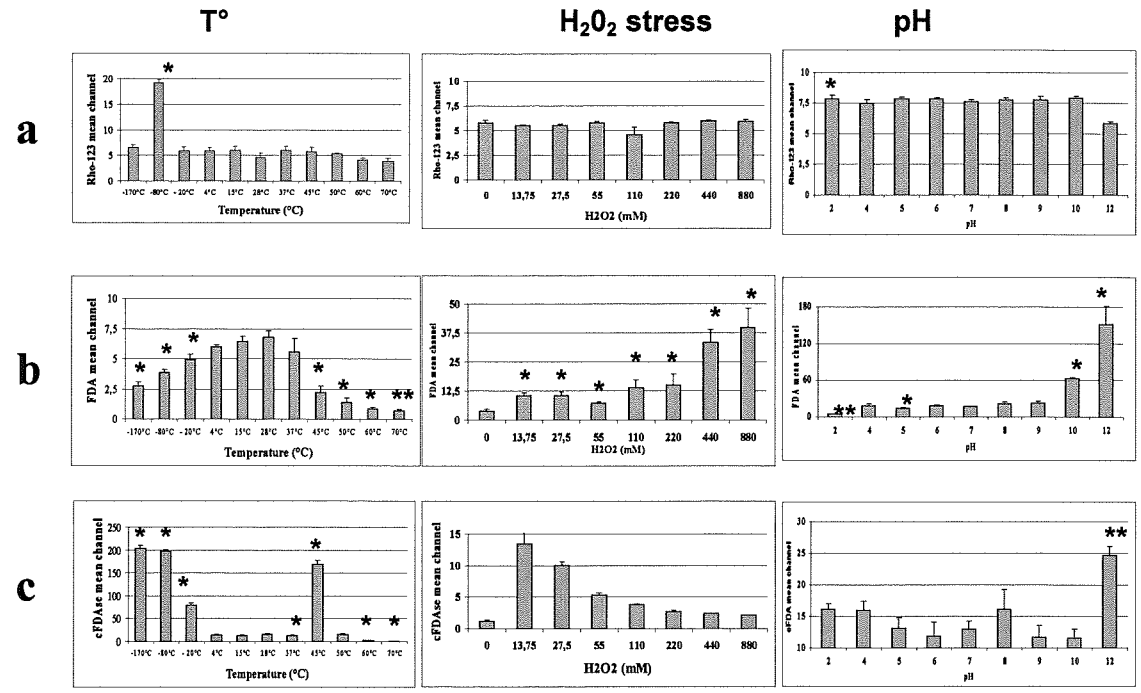



**Fig. 2.8. Effect of temperature, $H_2O_2$ or pH stress on *R. rubrum* on membrane potential (with Rho-123) (a), esterase activity (b) and intracellular pH (c). Results of the experiments represent triplicates ± SEM. * represent p<0.05; ** represents p<0.001.**

## 2.3.4. EFFECT OF TEMPERATURE, $H_2O_2$ OR PH STRESS ON ESTERASE ACTIVITY OF *R. RUBRUM.*

Figures 4, 5 and 6 (3rd row) as well as figure 8b show the effect of temperature, $H_2O_2$ or pH stress on esterase activity of *R. rubrum*. Following temperature stress, *R. rubrum* showed significant changes in esterase activity except at 4°C, 15°C and 37°C in comparison with control conditions (28°C). The oxidative stress induced on *R. rubrum* showed that the esterase activity was increased in function of the $H_2O_2$ concentration. Following extreme pH stresses (2, 5, 10 and 12), the esterase activity was significantly modified.

## 2.3.5. EFFECT OF TEMPERATURE, $H_2O_2$ OR PH STRESS ON INTRACELLULAR PH OF *R. RUBRUM.*

Figures 4, 5 and 6 (4th row) as well as figure 8c show representative histograms and statistical comparisons of cFDAse fluorescence changes following temperature, $H_2O_2$ or pH stress of *R. rubrum*. The application of a temperature stress showed an increase of intracellular pH at the three lowest (-170°C, -80°C and -20°C) and at almost all the highest temperatures (37°C, 45°C, 60°C and 70°C) studied. Oxidative stress induced no significant changes of intracellular pH. pH stress only induced a significant difference at pH 12.

## 2.3.6. EFFECT OF TEMPERATURE, $H_2O_2$ OR PH STRESS ON SUPEROXIDE ANION PRODUCTION OF *R. RUBRUM.*

Figures 4, 5 and 6 (5th row) and figure 9 show representative histograms and comparative graphs of HE fluorescence changes following temperature, $H_2O_2$ and pH stress of *R. rubrum*. *R. rubrum* showed a U curve pattern after temperature stress meaning that the superoxide anion production was increased at the lowest (-170°C and -80°C) as well as at the highest (45°C onwards) temperatures. Furthermore, *R. rubrum* showed an increased superoxide anion production from 110 mM $H_2O_2$ onwards. pH stress induced a significant difference of superoxide anion production at pH 4 and 12.

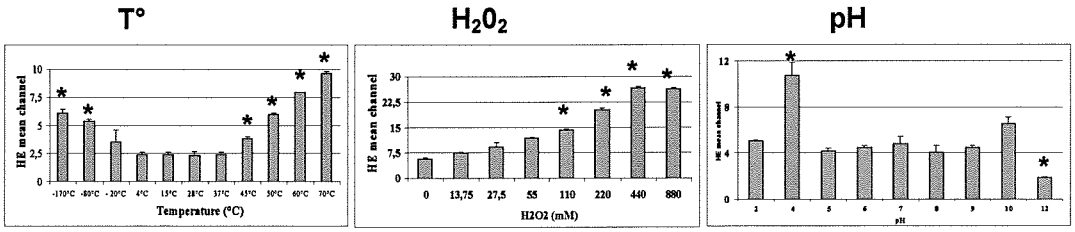



**Fig. 2.9. Effect of temperature, $H_2O_2$ or pH stress on *R. rubrum* on superoxide anion production. Results of the experiments represent triplicates ± SEM. * represent p<0.05; ** represents p<0.001.**

## 2.3.7. SUMMARY OF THE EFFECT OF TEMPERATURE, $H_2O_2$ OR PH STRESS ON *R. RUBRUM.*

The following three tables show the statistical summary concerning the effect of temperature and $H_2O_2$ on *R. rubrum* (one * represents a $p<0.05$ in comparison with the control (28°C in temperature experiments and 0 mM $H_2O_2$ in $H_2O_2$ experiments); two * represent $p < 0.001$).

| *T° (°C)* | *-170°C* | *-80°C* | *-20°C* | *4°C* | *15°C* | *37°C* | *45°C* | *50°C* | *60°C* | *70°C* |
|---|---|---|---|---|---|---|---|---|---|---|
| *Size* | | | | | | | * | * | * | |
| *Mbr. Perm.* | * | ** | * | | | | * | * | ** | * |
| *Mbr. Pot. (Dioc)* | ** | ** | * | * | * | * | ** | ** | * | ** |
| *Rho-123* | | | | | | | | | | |
| *Esterase* | * | * | * | | | | * | ** | ** | ** |
| *Activity* | | | | | | | | | | |
| *Intracellular pH* | ** | ** | ** | | | * | * | | ** | * |
| *Superoxide* | ** | ** | | | | | * | ** | * | ** |
| *anion production* | | | | | | | | | | |

| $H_2O_2$ (mM) | 13,25 | 27,5 | 55 | 110 | 220 | 440 | 880 |
|---|---|---|---|---|---|---|---|
| Size | * | * | * | * | * | * | * |
| Mbr. Perm. | * | * | * | * | * | * | * |
| Mbr. Pot. (Dioc) | | ** | * | ** | * | * | ** |
| Rho-123 | | | | | | | |
| Esterase | * | * | * | * | * | * | * |
| Activity | | | | | | | |
| Intracellular pH | | | | | | | |
| Superoxide | | | | * | * | ** | ** |
| anion production | | | | | | | |

As shown in the tables, the physiology of the cells did not seem to be altered between 4°C and 37°C. Above 37°C and under 4°C, *R. rubrum* appears to be damaged at the level of its membrane (as shown by the changes in membrane permeability and potential). Within the cytoplasm, enzymes, intracellular pH and ROS production are also affected to a level that could be explained by an induction of cell death at those temperatures. With regard to the effect of oxidative stress on *R. rubrum*, cells showed sensitivity to $H_2O_2$ concentrations from 13.25 mM meaning that an oxidative stress severely damages the physiology of *R. rubrum*.

## 2.4. Effect of temperature and $H_2O_2$ on Arthrospira sp. PCC8005.

### 2.4.1. EFFECT OF TEMPERATURE OR OXIDATIVE STRESS ON CELL SIZE AND GRANULARITY OF *ARTHROSPIRA SP*

Figures 10, 11a and 12a show the effect of temperature or oxidative stress on cell size of small and big filaments of *Arthrospira sp.*. Concerning the effect on cell size, a decrease of cell size in comparison with cells cultivated at 28°C could be noticed when cells were submitted to low (-170°C, -80°C, -20°C) or high (+50°C, 60°C and 70°C) temperatures. After oxidative stress, a decrease of cell size (in comparison with control conditions) in function of the concentration of $H_2O_2$ was observed in big filaments of *Arthrospira sp.* whilst no significant change was observed on small filaments of *Arthrospira sp.*.

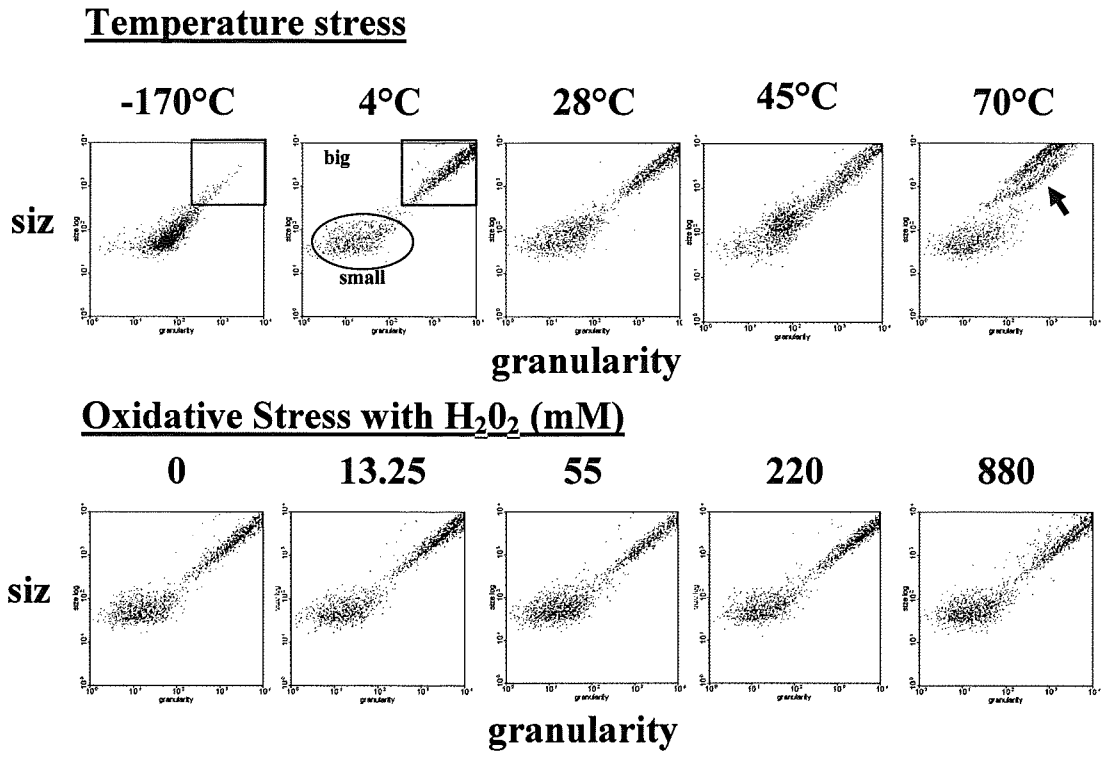



**Fig. 2.10. Representative dot-plots (side versus forward scatters) of *Arthrospira sp.* after a 1 hour incubation at either -170°C, 4°C, 28°C or 50°C or in the presence of 13.25, 110 or 880 mM $H_2O_2$.**

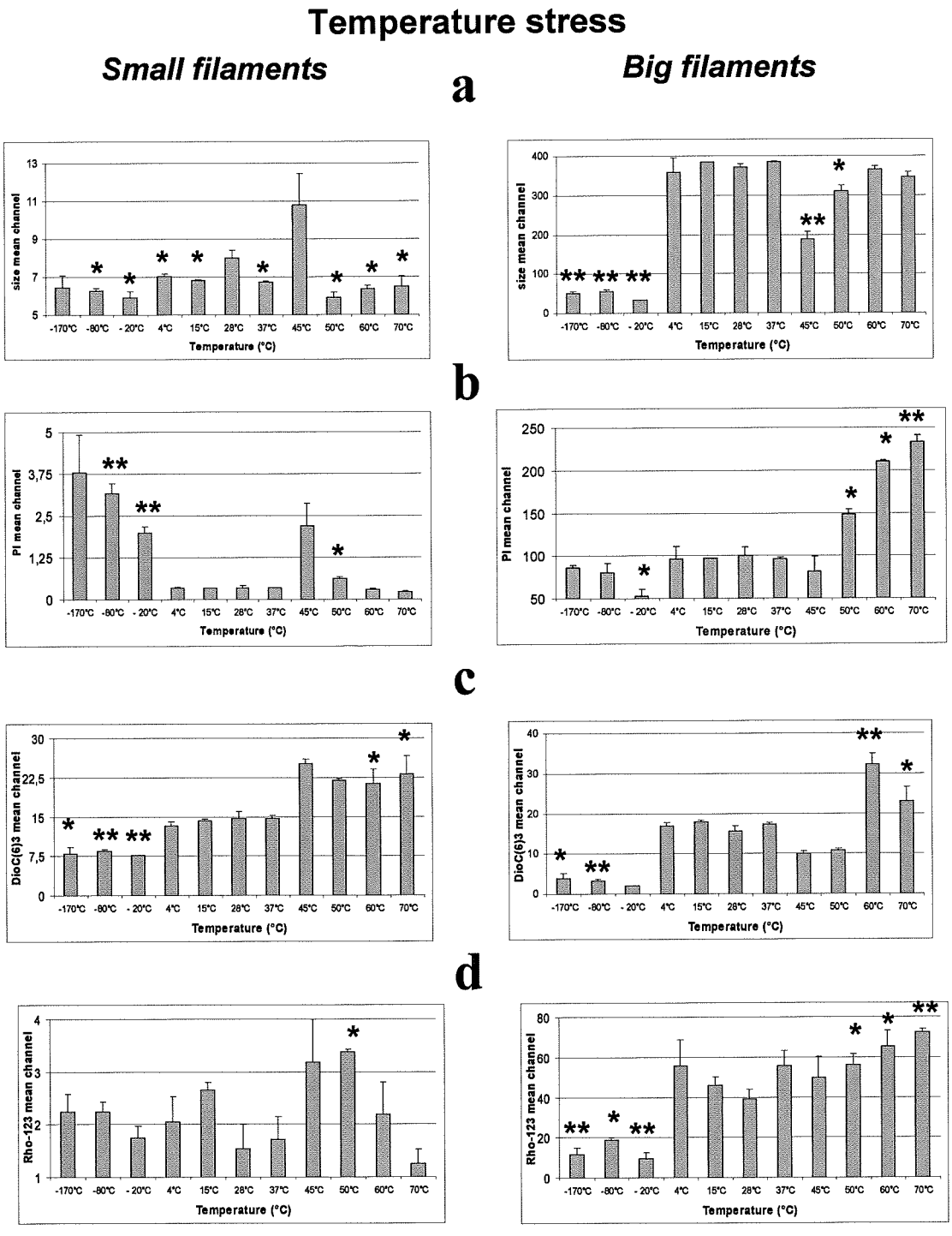



**Fig. 2.11. Effect of temperature stress on small (left column) and big (right column) filaments of Arthrospira sp. The effects of temperature are studied on cell size (a), membrane permeability (b) and membrane potential (c with DioC(6)3) and d with Rho-123). Results of the experiments represent triplicates ± SEM. * represent p<0.05; ** represents p<0.001.**

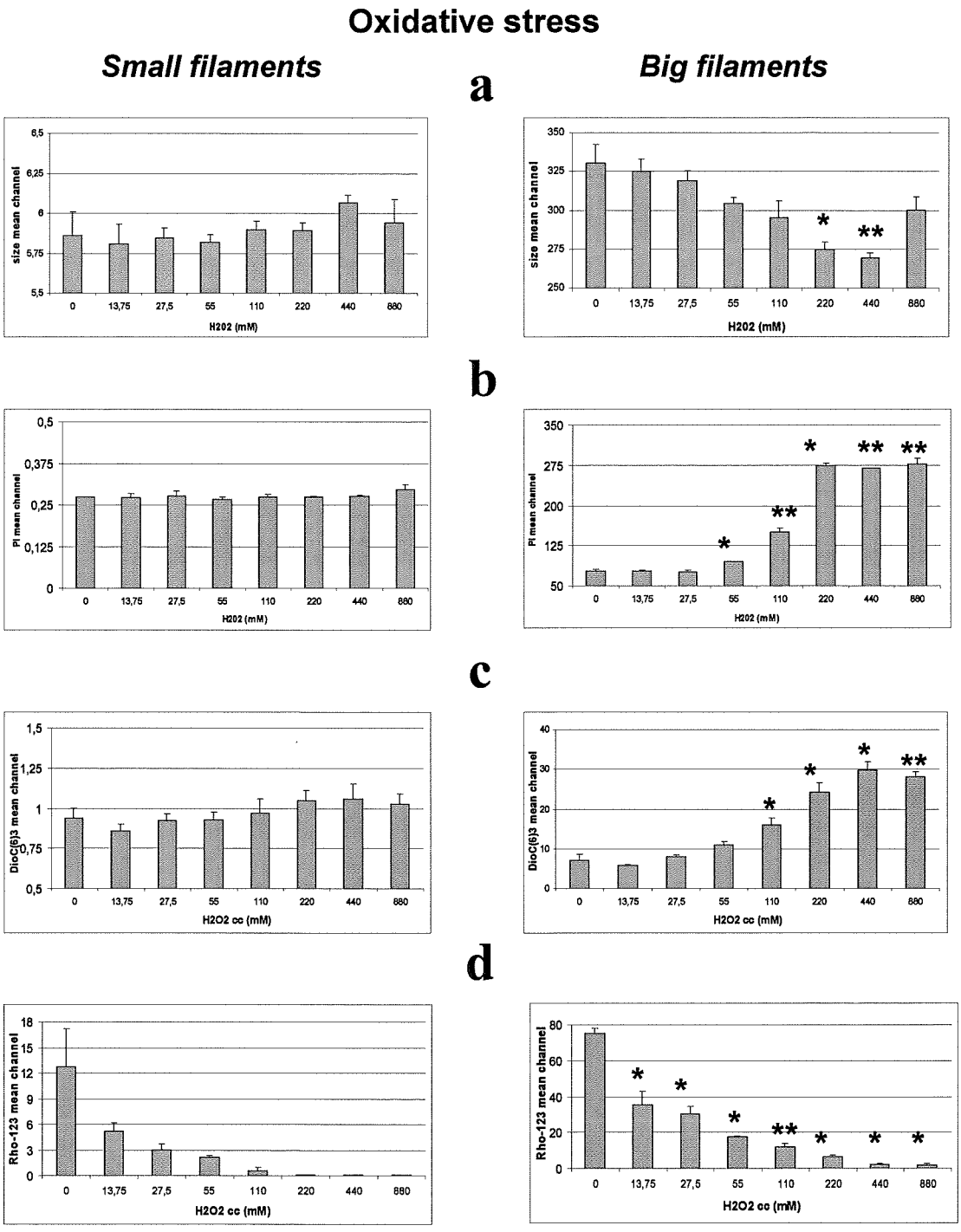



**Fig. 2.12. Effect of oxidative stress on *Arthrospira* sp. The effects of $H_2O_2$ are studied on on cell size (a), membrane permeability (b) and membrane potential (c with DioC(6)3 and d with Rho-123). Results of the experiments represent triplicates ± SEM. * represent p<0.05; ** represents p<0.001.**

## 2.4.2. EFFECT OF TEMPERATURE OR OXIDATIVE STRESS ON MEMBRANE INTEGRITY OF *ARTHROSPIRA SP.*

Figures 11b, 12b and 13 (1st row) show the effect of temperature or oxidative stress on membrane permeability of small and big filaments of *Arthrospira sp.* Small and big filaments reacted differently in the way that small filaments seemed to be more sensitive to low temperatures whereas big filaments reacted essentially to high temperatures. Oxidative stress induced an increase in membrane permeability that was clearly proportional to the concentration of $H_2O_2$ in the big filaments of *Arthrospira sp.*. Surprisingly, no change in membrane permeability was observed in the small filaments of *Arthrospira sp.* after oxidative treatment.

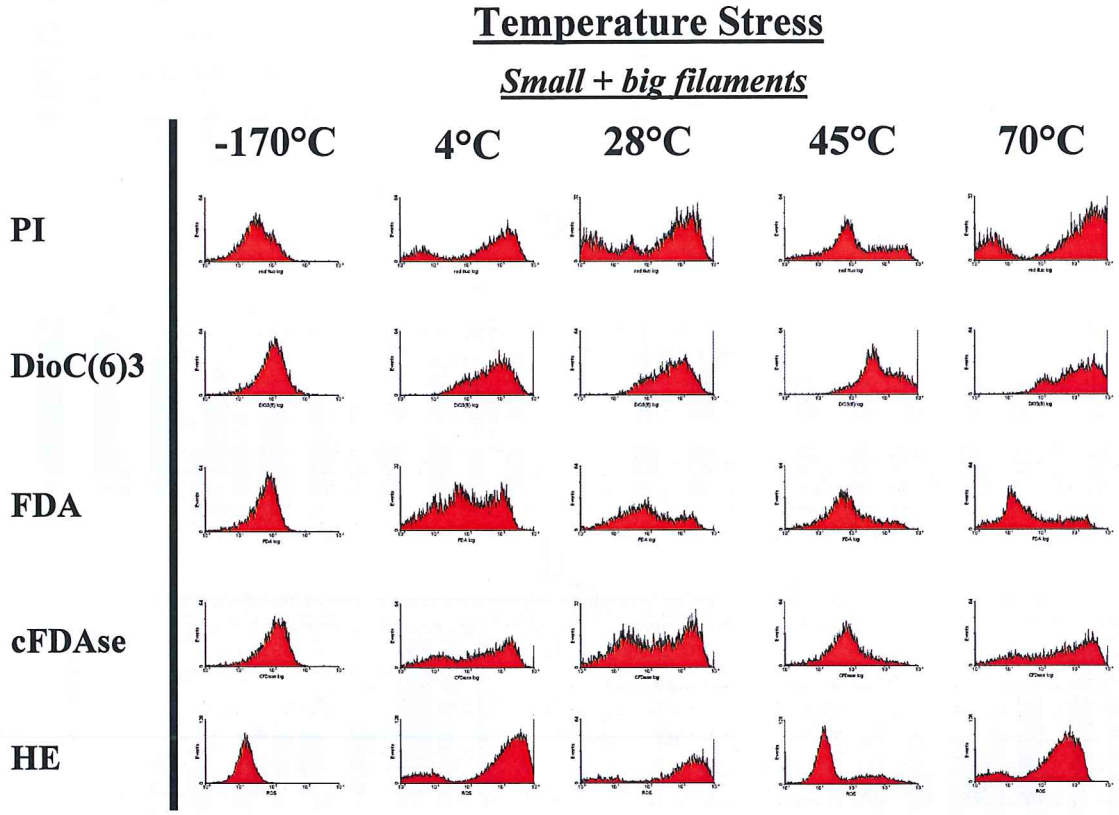



**Fig. 2.13. Representative histograms of PI (1st row), Dioc(6)3 (2nd row), FDA (3rd row), cFDase (4th row) and HE (5th row) fluorescences in *Arthrospira* (small and big filaments) following temperature stress.**

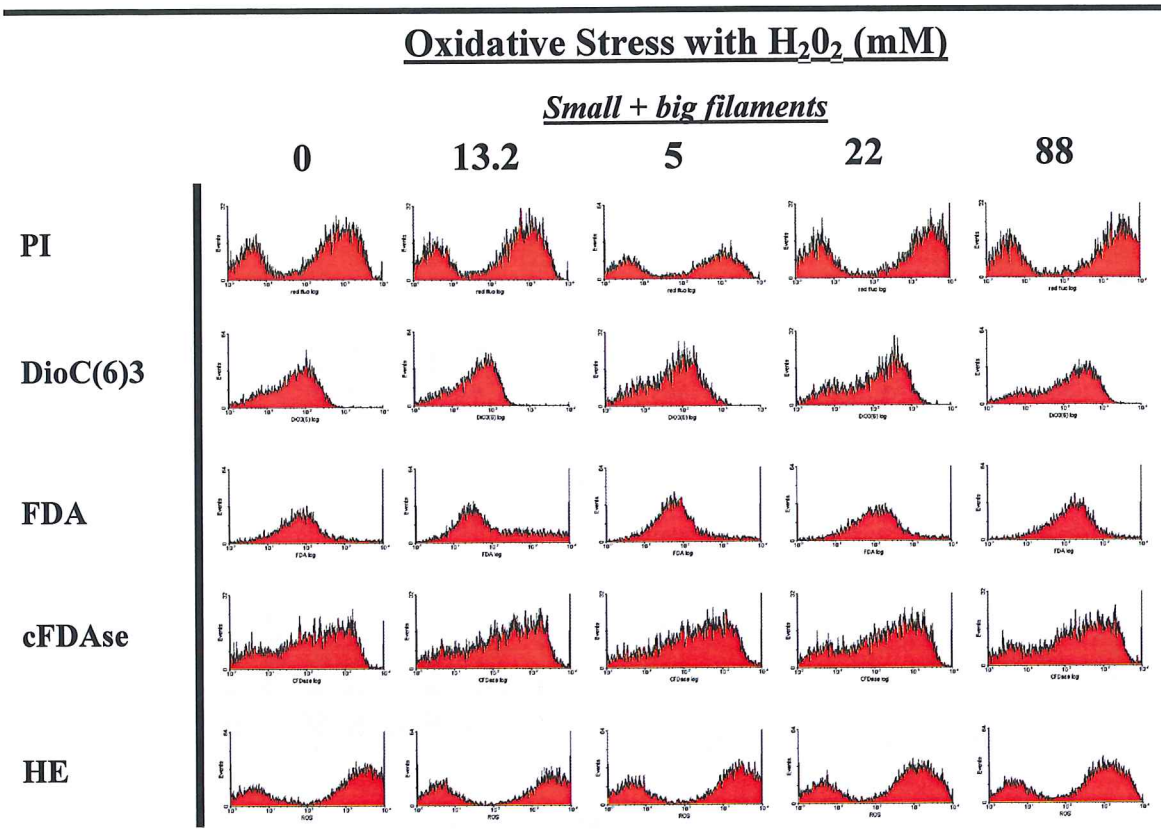



**Fig. 2.14. Representative histograms of PI (1st row), Dioc(6)3 (2nd row), FDA (3rd row), cFDase (4th row) and HE (5th row) fluorescences in *Arthrospira* sp. (small and big filaments) following oxidative stress.**

## 2.4.3. EFFECT OF TEMPERATURE OR OXIDATIVE STRESS ON MEMBRANE POTENTIAL OF *ARTHROSPIRA SP.*

Figures 11 (c and d), 12 (c and d)as well as 13 (2nd row) show the effect of temperature or oxidative stress on membrane potential of small and big filaments of *Arthrospira* sp.. Concerning *Arthrospira* sp. submitted to temperature stress, DioC(6)3 and Rho-123 stainings showed that for small and big filaments of *Arthrospira* sp., the membrane potential was lowered after cold treatments (-170°C, -80°C and -20°C) and increased after heat treatment (+50°C, +60°C and 70°C) except that no significant change after temperature treatment could be observed following Rho-123 staining in small filaments. Concerning the changes induced by oxidative stress, *Arthrospira* sp. (small + big filaments) stained with DioC(6)3 showed an increase of membrane potential in function of $H_2O_2$ concentration. In contrast, Rho-123 staining of *Arthrospira* sp. showed a decrease of fluorescence inversely proportional to the $H_2O_2$ concentration.

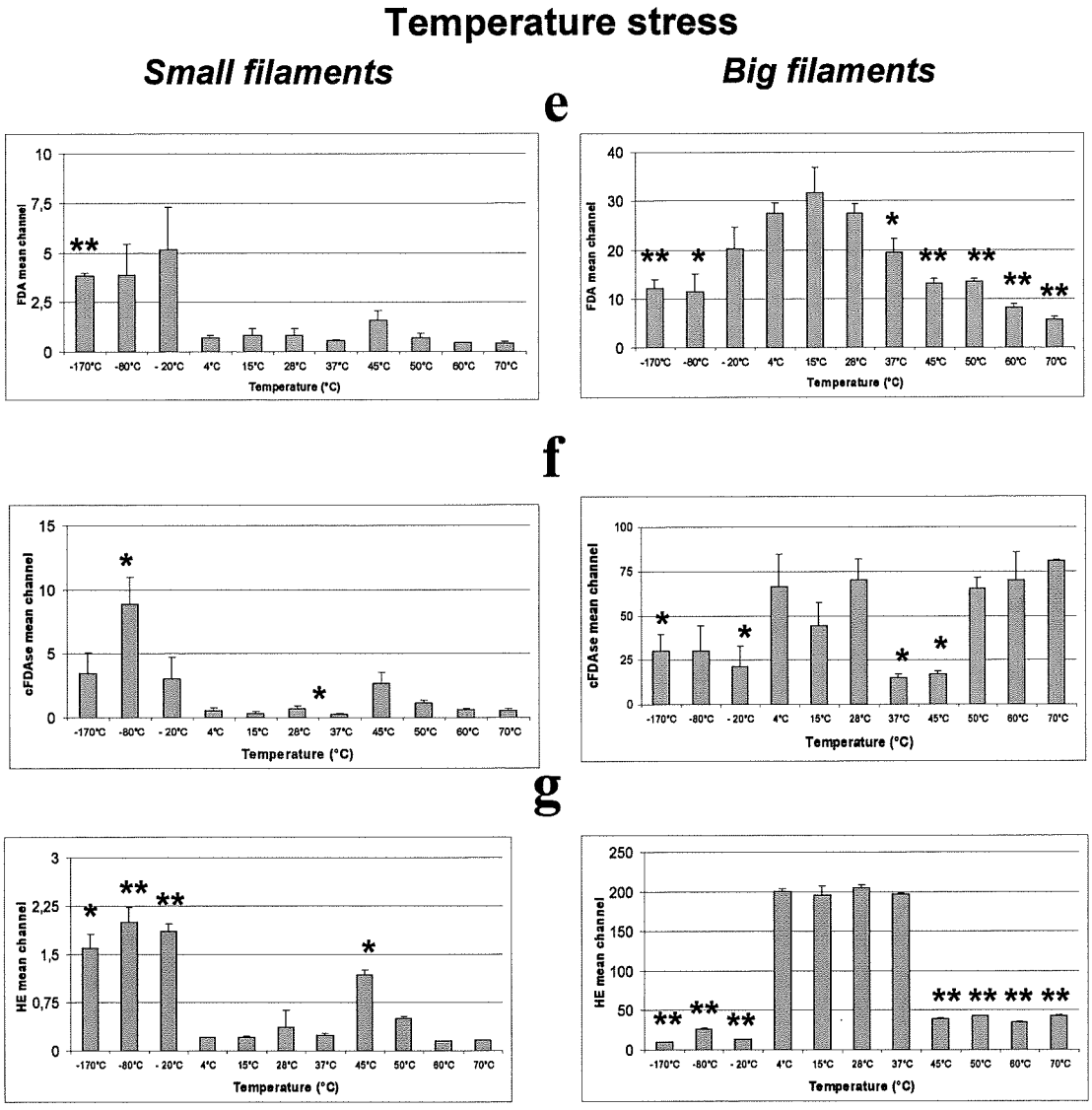



**Fig. 2.15. Effect of temperature stress on small (left column) and big (right column) filaments of A. platensis. The effects of temperature are studied on esterase activity (e), intracellular pH (f) and superoxide anion production (g).**

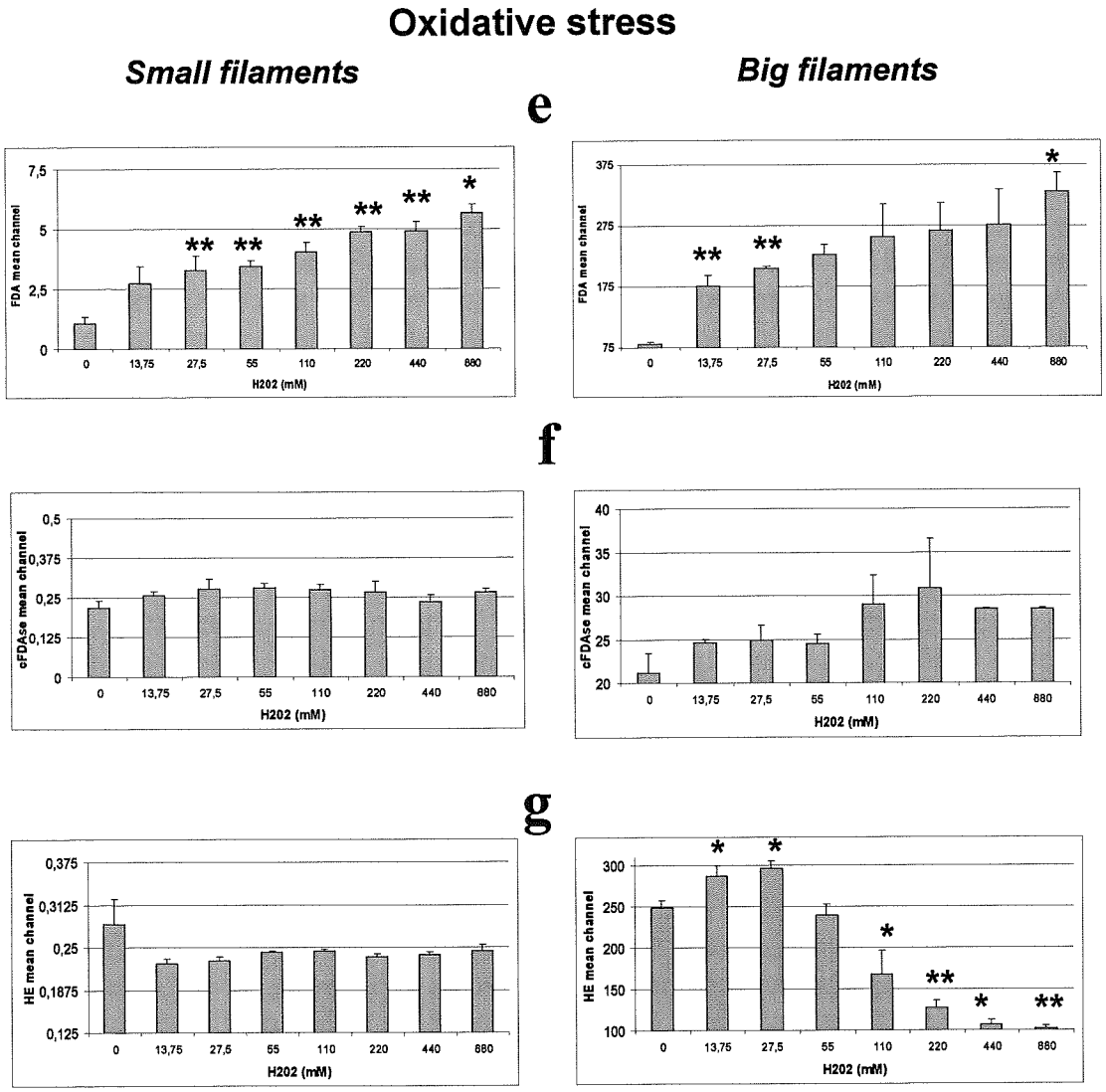



**Fig. 2.16. Effect of oxidative stress on small (left column) and big (right column) filaments of A. platensis. The effects of temperature are studied on esterase activity (e), intracellular pH (f) and superoxide anion production (g).**

## 2.4.3. EFFECT OF TEMPERATURE OR OXIDATIVE STRESS ON ESTERASE ACTIVITY OF *ARTHROSPIRA SP.*

Figures 14 (3rd row), 15e and 16e show the effect of temperature or oxidative stress on esterase activity of small and big filaments of *Arthrospira* sp.. Following temperature stress, the big filaments of *Arthrospira* sp. showed a Gaussian pattern with a maximum of esterase activity at physiological temperature (28°C) whereas the temperature stress on the small filaments of *Arthrospira* sp. showed an increase of esterase activity after freezing (-170°C, -80°C and -20°C) and after 45°C. The oxidative stress induced on small and big filaments of *Arthrospira* sp. showed that the esterase activity was increased in function of the $H_2O_2$ concentration.

## 2.4.4. EFFECT OF TEMPERATURE AND OXIDATIVE STRESS ON INTRACELLULAR PH OF *ARTHROSPIRA SP.*

Figures 14 (4th row), 15f and 15g show the effect of temperature (1if and 2if) or oxidative (3if and 4if) stress on intracellular pH of small and big filaments of *Arthrospira* sp..

The intracellular pH of the small filaments of *Arthrospira* sp. was only affected by the freezing temperatures and by an incubation at 45°C whereas the intracellular pH of big filaments of *Arthrospira* sp. was decreased after freezing and at 37°C and 45°C. The oxidative stress induced some changes of intracellular pH as well. Indeed, in *Arthrospira* sp., a slight increase of CFDAse is observed in function of $H_2O_2$ concentration.

## 2.4.5. EFFECT OF TEMPERATURE OR OXIDATIVE STRESS ON SUPEROXIDE ANION PRODUCTION OF *ARTHROSPIRA SP.*

Figures 14 (5th row), 15g and 16g show the effect of temperature or oxidative stress on intracellular pH of small and big filaments of *Arthrospira* sp.. Small and big filaments of *Arthrospira* sp. did not react the same way following temperature stress. Indeed, small filaments produced significantly more superoxide anions at freezing temperatures and at 45°C and 50°C whereas the production of superoxide anions in big filaments was lower following freezing (-170°C, 80°C and -20°C) and high (45°C, 50°C, 60°C and 70°C) temperatures.

## 2.4.6. SUMMARY OF THE EFFECT OF TEMPERATURE OR OXIDATIVE STRESS ON *ARTHROSPIRA SP.*

The following three tables show the statistical summary concerning the effect of temperature and $H_2O_2$ on *Arthrospira sp.* (one * represents a $p<0.05$ in comparison with the control (28°C in temperature experiments and 0 mM $H_2O_2$ in $H_2O_2$ experiments); two * represent $p < 0.001$). As shown in the following tables, the physiology of the cells did not seem to be higly altered between 4°C and 37°C in small as well as big filaments. Above 37°C and under 4°C, *Arthrospira sp.* appears to be damaged at the level of its membrane (as shown by the changes in membrane permeability and potential). At those temperatures, within the cytoplasm, enzymes, intracellular pH and ROS production are also affected to a level that could be explained by an induction of cell death at those temperatures. With regard to the effect of oxidative stress on *A. sp.*, small filaments were quite resistant to oxidative stress whilst big filaments were already damages at the lowest concentration of 13.25 mM meaning that an oxidative stress severely damages the physiology of big filaments of *Arthrospira sp.*

**Small filaments :**

| *T° (°C)* | *-170°C* | *-80°C* | *-20°C* | *4°C* | *15°C* | *37°C* | *45°C* | *50°C* | *60°C* | *70°C* |
|---|---|---|---|---|---|---|---|---|---|---|
| *Size* | | * | * | * | * | * | | * | * | * |
| *Mbr. Perm.* | | ** | ** | | | | | * | | |
| *Mbr. Pot. (Dioc)* | * | ** | ** | | | | | | * | * |
| *Rho-123* | | | | | | | | * | | |
| *Esterase Activity* | * | | | | | | | | | |
| *Intracellular pH* | | * | | | | | | | | |
| *Superoxide anion production* | * | ** | ** | | | | * | | | |

**Big filaments :**

| *T° (°C)* | *-170°C* | *-80°C* | *-20°C* | *4°C* | *15°C* | *37°C* | *45°C* | *50°C* | *60°C* | *70°C* |
|---|---|---|---|---|---|---|---|---|---|---|
| *Size* | ** | ** | ** | | | | ** | * | | |
| *Mbr. Perm.* | | | * | | | | | * | * | ** |
| *Mbr. Pot. (Dioc)* | * | ** | | | | | | | ** | ** |
| *Rho-123* | ** | * | ** | | | | | * | * | ** |
| *Esterase* | ** | * | | | | * | ** | ** | ** | ** |
| *Activity* | | | | | | | | | | |
| *Intracellular pH* | * | | * | | | * | * | | | |
| *Superoxide* | ** | ** | ** | | | | ** | ** | ** | ** |
| *anion production* | | | | | | | | | | |

**Small filaments :**

| *$H_2O_2$ (mM)* | *13,25* | *27,5* | *55* | *110* | *220* | *440* | *880* |
|---|---|---|---|---|---|---|---|
| *Size* | | | | | | | |
| *Mbr. Perm.* | | | | | | | |
| *Mbr. Pot. (Dioc)* | | | | | | | |
| *Rho-123* | | | | | | | |
| *Esterase* | | ** | ** | ** | ** | ** | ** |
| *Activity* | | | | | | | |
| *Intracellular pH* | | | | | | | |
| *Superoxide* | | | | | | | |
| *anion production* | | | | | | | |

**Big filaments :**

| $H_2O_2$ (mM) | 13,25 | 27,5 | 55 | 110 | 220 | 440 | 880 |
|---|---|---|---|---|---|---|---|
| Size | | | | | * | ** | |
| Mbr. Perm. | | | ** | ** | ** | ** | ** |
| Mbr. Pot. (Dioc) | | | | * | ** | ** | ** |
| Rho-123 | ** | ** | ** | ** | ** | ** | ** |
| Esterase | ** | ** | | | | | * |
| Activity | | | | | | | |
| Intracellular pH | | | | | | | |
| Superoxide | * | * | | * | ** | ** | ** |
| anion production | | | | | | | |

## 2.5. Discussion

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

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

and potential. Following oxidative stress, the membrane permeability and potential increased in function of the concentration of $H_2O_2$.

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

## *2.6. Presentations*

S. Baatout, P. De Boever, F. Marty, L. Hendrickx, M. Mergeay, "Flow cytometric study of membrane permeability in six bacterial strains under stress conditions (temperature, X- and UV-irradiations)", Annual meeting of the Belgian Society of Microbiology, Brussels, 22/11/2002.

S. Baatout, P. De Boever, H. Derradji, F. Marty, L. Hendrickx, M. Mergeay, "Assessment of oxidative stress on superoxide anion production, membrane permeability, size and granularity of four bacterial strains by flow cytometry", Annual Symposium of the Belgian Society of Cytometry, Namur, 15/11/2002.

S. Baatout, R. Wattiez, L. Heindrickx, F. Marty, M. Mergeay, "Changes associated with temperature, $H_2O_2$ and pH stresses on the physiology of *R. rubrum",* Annual Symposium of the Belgian Society of Cytometry, Leuven, 07/11/2003.

S. Baatout, P. De Boever, M. Mergeay, "Temperature-induced changes on bacterial physiology", Annual Symposium of the Belgian Society of Cytometry, Leuven, 07/11/2003.

S. Baatout, M. Deruelle, H. Derradji, M. Mergeay, P. Van Oostveldt, S. Bekaert, "Cytotoxicity of spirulina extracts on human cancer cell lines", Annual Symposium of the Belgian Society of Cytometry, Leuven, 07/11/2003.

R. Wattiez, S. Baatout, L. Hendrickx, F. Marty, M. Mergeay, "Changes associated with temperature or $H_2O_2$ stress on the physiology of *A. platensis",* Annual Symposium of the Belgian Society of Microbiology, Brussels, 21/11/2003.

P. De Boever, S. Baatout, M. Mergeay, "Physiological changes induced in bacteria following oxidative stress", Annual Symposium of the Belgian Society of Microbiology, Brussels, 21/11/2003.

S. Baatout, M. Deruelle, H. Derradji, M. Mergeay, P. Van Oostveldt, S. Bekaert, "Cytotoxicity of spirulina extracts on human cancer cell lines", Annual Symposium of the Belgian Society of Microbiology, Brussels, 21/11/2003.

Publications in preparation:

S. Baatout, R. Wattiez, L. Hendrickx, F. Marty, M. Mergeay. Changes associated with temperature, $H_2O_2$ and pH stresses on the physiology of *R. rubrum* ATCC25903.

S. Baatout, P. De Boever, M. Mergeay. Temperature-induced physiological changes estimated by flow cytometry in four bacterial strains.

P. De Boever, S. Baatout, M. Mergeay. Physiological changes induced in four bacterial strains following oxidative stress.

# 3 PROTEOMIC ANALYSIS OF THERMIC, OXIDATIVE AND PH STRESS ON *RHODOSPIRILLUM RUBRUM* ATCC25903.

## *3.1. Introduction*

A proteomic approach based on two-dimensional gel separation and mass spectrometry protein identification was used to detect and identify protein modifications in different stress conditions (temperature and pH variation and oxidative stress). Firstly, we needed to built 2-DE maps from these both bacteria with the same protein extraction method as *Ralstonia.* Afterwards, proteins were identified using MALDI-TOF-MS analysis.

## *3.2. 2-DE proteomic mapping of R. rubrum ATCC25903*

As shown in figure 9, the two dimensional gel electrophoresis maps obtained from total protein extracts of *Rhodospirullum rubrum* (center) ATCC25903 was qualitatively very different from the 2-DE map of *Ralstonia metallidurans* (right) extract. The 2-DE maps obtained from *Rhodospirillum* extracts were characterised by a lot of high concentrated proteins probably corresponding to the proteins involved in the photosynthetic system.

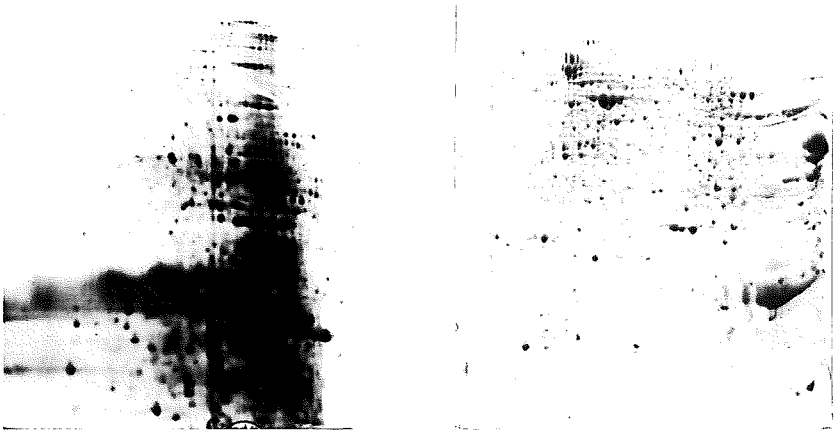


**Fig. 3.1. Comparison of 2-DE maps of total protein extracts from *Rhodospirullum rubrum* ATCC25903 (center) and *Ralstonia metallidurans* CH34 (left). The protein were extracted with the buffer containing 8M Urea, 4% (w/v) CHAPS, 40mM Tris, 0.2% (v/v) Pharmalyte 3-10, 2mM Tributyl Phosphine (Bio-Rad) , ¼ tablet/ml Complete Mini EDTA Free Protease Inhibitor Cocktail (Roche)) and further lysed for 1 minute in an Elma Transsonic 450/H sonicator at 4°C. The protein were separated by two dimensional gel electrophoresis using a 3-10 IPGs in the first dimension step.**

This was more obviuosly observable around the region of 20KDa protein mass for *Rhodospirillum* extracts, respectively. In this context, a poor protein separation for these samples has been obtained.

Nevertheless, in the case of *Rhodospirullum*, the two dimensional gel elctrophoresis from total protein extraction presented a reproducibility that allowed a reliable comparison of the proteomic profile between the bacterial samples treated under either control or under stress conditions as a temperature variations and a oxidatifstress (comparison by a bioinformatic system as PDQUEST). Also in the case of *Rhodospirillum* proteomic profiles of the bacterial suspension undergoing thermic stress (43°C, 2h25min) as well as oxidative stress (50um $h_2O_2$, 1h25min, room temperature) was compared with controlled conditions. The Bioinformatic analysis of the protein spots allowed the detection of increased intensity of three spots under thermic stress. Four increased intensity spots were observed on the profile obtained from the suspension that had undergone oxidative stress. These protein spots have been identified by mass spectrometry in tandem (ESI-MS-MS; TN70.9), the identity of the proteins was obtained by comparison of the obtained peptide sequences with known proteins on the NCBI database (table 2).

| Protein name | Stress | Induction/ Repression | Peptide sequence | Gene number | Contig |
|---|---|---|---|---|---|
| **GroES** | Thermic<br>Oxidative | Ind<br>Ind | VVALDVK<br>TAGGIIIPDTAK | 2070 | 14 |
| **GroEl** | Thermic | Ind | VGNEGVITVEEAK<br>LENVTIDMLGTAK | 2071 | 14 |
| **Thioredoxin** | Oxydative | Ind | INIDENPQTPSK | 3170 | 2 |
| **DnaK** | Thermic | Ind | SQTFTAEDSQTAVTIR | 315 | 1 |
| **RecA** | Oxydative | Ind | SGAIDVLVIDSVAALVP | 3157 | 2 |
| **HtpG** | Thermic<br>OxYdative | Ind<br>Rep | LSIDAEAGTLTIADNGIGMNR | 35 | 1 |
| **Alkyl hydroperoxy de reductase subunit C** | OxYdative | Ind | NFDVLIEDAGLADR | 6609 | 7 |

**Table 3.1. Peptide sequences obtained by ESI-MS-MS and homology with corresponding genes on the genome sequence of *Rhodospirillum* ATCC11170 induced or repressed during thermic or oxidative stress.**

The protein spots that showed significant differences in intensity (3 different extracts from 3 separated experiments) were identified. Their protein sequence is listed below (the experimental sequenced peptides are highlighted in red). Interestingly, in other bacteria more of these proteins were known to be modified during temperature or oxidative stress.

**GroES**

MKFRPLHDRVLVKRLEGEEKTAGGIIIPDTAKEKPMEGEVVAVGSGARGDDGKVVALDVKAGDRILFG
KWSGTEVKIDGTDFLIMKESDIMGIVA

**GroEL**

MAAKDVKFSTDARDRLLRGVDILANAVKVTLGPKGRNVVLDKSYGAPRITKDGVSVAKEIELKDKFEN
MGAQMVKEVASKSADVAGDGTTTATVLAQAIVREGVKSVAAGMNPMDLKRGIDLAVLAVVEDVKKR
SKKIKTSAEVAQVGTISANGDEEVGKIIATAMEKVGNEGVITVEEAKGLDTELDVVEGMQFDRGYLSPY
FVTNAEKMVADLENPYILLHEKKLSGLQALLPVLEAVVQSSRPLLIIAEDVEGEALATLVVNKLRGGLKV
AAVKAPGFGDRRKAMLEDIAILTGGQVISEDLGIKLENVTIDMLGTAKKVTITKEETTLVDGAGDKKDIE
ARCSQIRANIEDTSSDYDREKLQERLAKLAGGVAVIKVGGATETEVKEKKDRVDDAMHATRAAVEEGV

VAGGGVALLHAIRSLDSVKGANPDQNVGIEIVRRALQAPVRQIAENAGVDGAVVAGKLLENSDTDFGY
NAQTGIYENLVTAGVIDPTKVVRAALQGAASIAGLLITTEAMVAEIPEKKDAMPSPDMGGMGGMGGMG
F

**THIOREDOXIN**

MKQVSDASFEEDVLKADGPVLVDFWAEWCGPCRQIAPALEELATALGDKVTVAKINI
DENPQTPSKYGVRGIPTLMIFKDGQVAATKIGALPKTKLFEWVEASV

**DNAK**

LQKMKETAEAHLGEPVTQAVITVPAYFNDSQRQATKDAGKIAGLEVLRIINEPTAAALAYGMDKKNTGT
IAVFDLGGGTFDVSVLEIGDGVFEVKSTNGDTFLGGEDFDARIIDYLASEFKKEQGIDLRTDRLALQRLKE
AAEKAKIELSSSMQTEVNLPFITADQAGPKHLNIKLTRAKLEALVDDLVQRTVEPCRKALADAGIKASEI
DEVILVGGMTRMPKVQQVVKDFFGREPHKGVNPDEVVAMGAAIQGGVLKGDVKDVLLLDVTPLSLGIE
TLGGVFTRLIDRNTTIPTRKSQTFSTAEDSQTAVTIRVFQGEREMAADNKMLGQFDLVGLPSAPRGVPQIE
VTFDIDANGIVNVSAKDKATGKEQAIRIQASGGLSDNDIERMVKEAELNAEADRKRKEAVEARNHADGL
IHATEKNLKEYGDKIPAEDKAKVEGDLTALKAVLDSEDAESIKAKTDALMQSAMKLGEAAYSAGQSAE
GAPHAAGAEASAQSRTDDGVVDADFEEVDEKKGH*

**RecA**

MSQSVLRLVDKDTMDKQKALEAAVGQIERAFGKGSIMKLGQRGSVVDIESISTGSLGLDIALGIGGLPRG
RIVEIYGPESSGKTTLALHVVAEAQKKGGQCAFVDAEHAFDPLYARKLGVSLDDLLVSQPDTGEQALEI
ADTLVRSGAIDVLVIDSVAALVPKAELEGDMGDSHVGLQARLMSQALRKLTGTVSRSNTLIIFINQIRMKI
GVMFGNPETTTGGNALKFYASVRLDIRRIGAVKDKEEVVGNQTRVKVVKNKVAPPFKVVEFDIMYGEG
ISKLGEMLDLGVKANIIEKSGAWFSYNSTRIGQGRENAKQFLRDNPAMAEEIENAVRANAGLIAEEMIGG
PGGEDDDAGGAAGVGDEA

**HPTG**

MSEETLSFQAEVSKLLDIVVHSLYSDRKIFLRELISNASDACDKLRYEGLTQPALLEGDGAFRIRLSIDAEA
GTLTIADNGIGMNRHELIENLGTIARSGTQAFAEALKAKSQAASGDVSLIGQFGVGFYSAFMVADKVEV
VTRRAGEAQGWRWSSDGKGSFSVSEVEGAGRGAAITLHLREDARDFLDEHRLREIVKTYSDHIAIPVDY
AGKEGEPERLNEASALWTRPRDQITDE

These preliminary results show that the proteomic approach was an appropriate method to detect gene induction or repression in the MELiSSA strains. Nevertheless, the poor separation obtained after two-dimensional gel electrophoresis does not allow a statistical comparison on the whole proteome especially for the protein characterised by a lower concentration. Firstly, due to the sensitivity of this technology, it is important to take extra measures in sample preparation. If comparative analyses are desired, it is essential that variables between samples are limited to controlled experimental modifications. For this reason, all samples that will be used for comparative analysis should be prepared in an identical manner. It is also necessary, prior to sample submission, to clean up the protein samples, *i.e.* remove cell debris and other latent particulates.

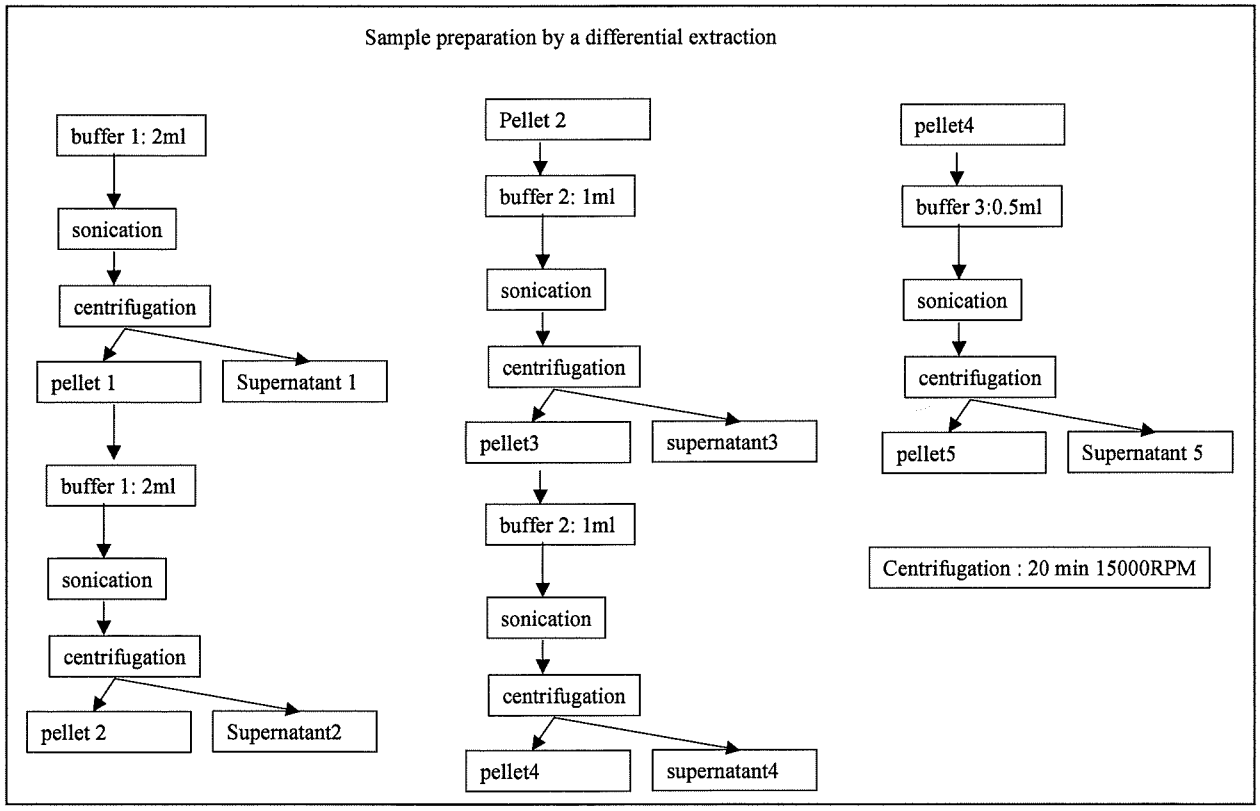



**Fig. 3.2. Sample preparation by differential extraction. Buffer 1: Tris-Hcl 50mM pH 7.5. Buffer 2: 8M Urea, 4% (w/v) CHAPS, 40mM Tris, 0.2% (v/v) Pharmalyte 3-10, 2mM Tributyl Phosphine (Bio-Rad). Buffer 3: containing 5M Urea, 2 M Thiourea, SB 3-10 (v/v), 4% (w/v) CHAPS, 40mM Tris, 0.2% (v/v) Pharmalyte 3-10, 2mM Tributyl Phosphine (Bio-Rad).**

This clean up is essential and will greatly improve proteomic results. Secondly, if the initial extraction and clean up results in a whole cell extracts, prefractionation of whole extracts is strongly recommended. Prefractionation aids analysis by reducing the spot pattern complexity for individual gels while increasing the total protein detection for the entire protein sample.

There are a number of methods of prefractionation, *e.g.*, fractionation based on hydrophobicity or salt precipitation, are two examples. The type of prefractionation method used is dependent on the sample type. One remedy is to use of multiple overlapping narrow IPGs in the first dimension (zoom-in gels) and/ or used prefractionation proteins by isolation of cell compartments (extracellular, cytoplasmic, periplasmic) and by sequential extraction procedures (membranes proteins) with increasingly powerful solubilizing buffer (usually aqueous buffers, organic solvents and detergent-based to extraction solutions). In this context, we have tested different sample preparation to increase the resolution during the electrophoresis especially by a sequential extraction procedure using different buffers containing different detergents (buffer 1, buffer 2 and buffer 3).

Sequential extractions are being utilised to distinguish between soluble (cytoplasmic and periplasmic) and non-soluble proteins (membrane-associated proteins). As shown by the schema of the differential extraction (Figure 3.2.), for each buffer, two extractions were realised in the aim to obtain a quantitatively extraction.

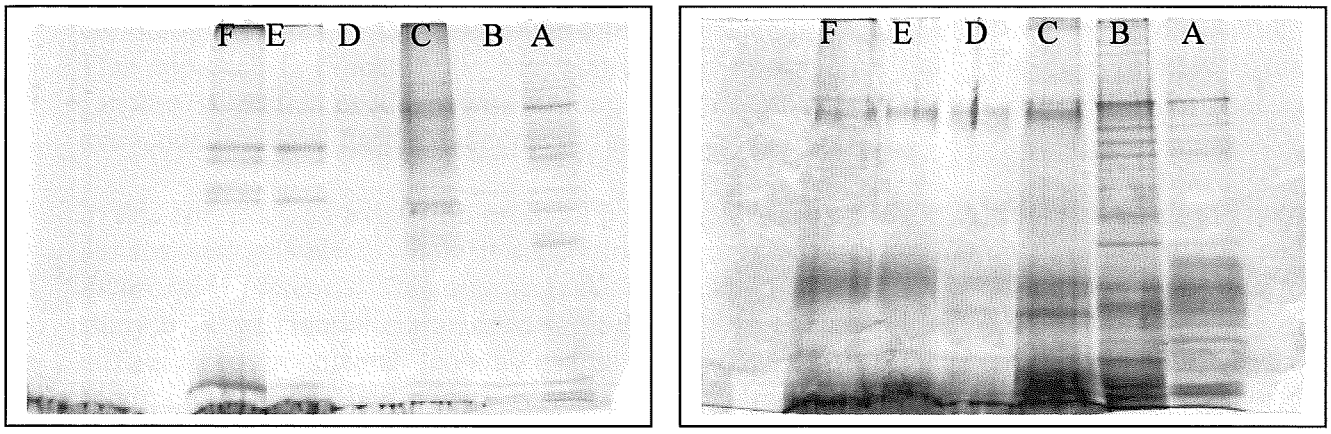



**Fig. 3.3. SDS-PAGE of the different supernatants obtained during the differential extractions from *Rhodospirullum rubrum* (left) and *Arthrospira platensis* (right). *A: supernatant 1; B: supernatant 2; C: supernatant 3; D: supernatant 4; E: supernatant 6* from *Rhodospirullum rubrum.***

After each extraction, supernatants containing proteins were analysed by SDS-PAGE. For *Rhodospirillum*, the extraction is quantitative for each buffer except for the buffer 3. Indeed, the protein concentration present in the supernatant 3 and 4 is quantitatively lower than the one in the supernatant 1 and 2 (figure 3.3.).

To increase the lysis of the bacteria, each extraction is realised with a sonication step (5 min at $4^0$C). The protein extraction without this sonication step was not quantitatively, especially with the buffer 1 containing no detergent (data not shown). Moreover, the protein spectrums in the different supernatants were different in agreement with a differential extraction.

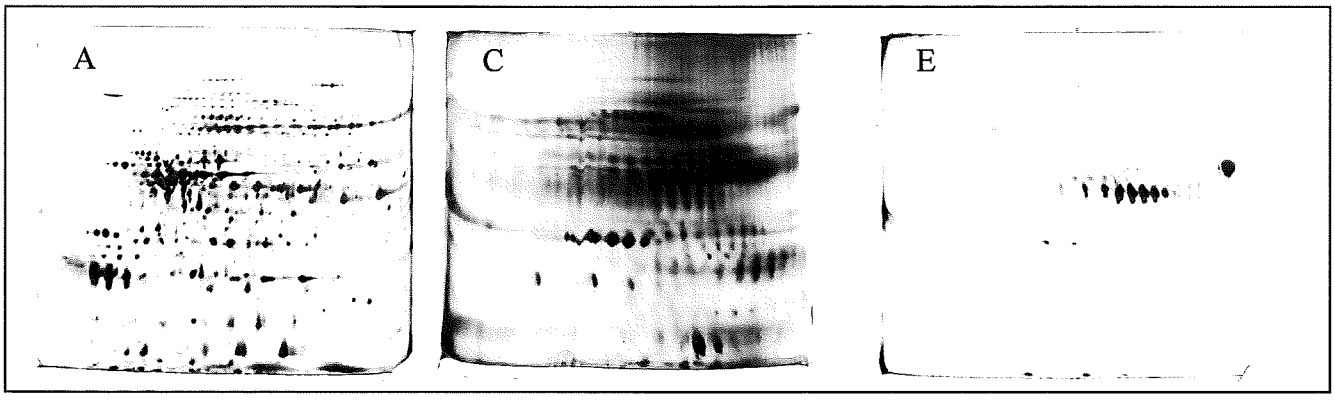



**Fig. 3.4. 2-DE of the different supernatants obtained during the differential extraction from *Rhodospirullum rubrum* (left) and *Arthrospira platensis* (right). *A: supernatant 1; B: supernatant 2; C: supernatant 3; D: supernatant 4; E: supernatant 6* from *Rhodospirullum rubrum.***

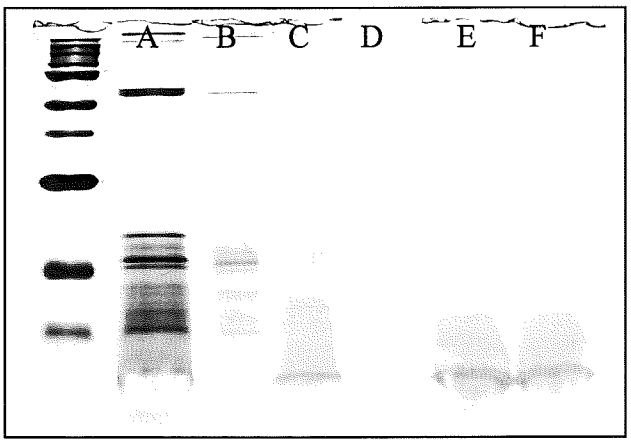



**Fig3.5. SDS-PAGE of the different supernatants obtained during the differential extraction from *Arthrospira platensis*. *A: supernatant 1; B: supernatant 2; C: supernatant 3; D: supernatant 4; E: supernatant 5; F: supernatant 6.***

The resolution of the separation of the three 2-DEs obtained with the differential extraction is better than one obtained without differential extraction and to allow now a quantitative detection of the individual spots by a bioinformatic system. The supernatant A, B and C probably were constitute essentially of cytoplasmic soluble protein, of the membrane proteins and of the membrane proteins characterised by a high hydrophobicity, respectively In contrast, any differential extraction from *Arthrospira platensis* extracts has been obtained in spite of the sonication step (Figure 3.4.). In contrast with *Rhodospirillum*, after a sonication step in TRIS-HCl buffer without detergent (Buffer 1), the lysis of the bacteria was incomplete: The protein concentration in the supernatant 1 was similar to supernatant 2. To increase the mechanical lysis, the sonication step has been replaced by a glass beads lysis (15 min. at room temperature). As shown in the figure 3.5., this step allows a quantitative lysis of *Arthrospira.*

## *3.3. Conclusions*

With the DNA sequences of more genomes completed, as well as a draft sequence of *Ralstonia metallidurans* CH34 and *Rhodospirillum rubrum* genomes, a major challenge in modern biology is to understand the expression, function, and regulation of the entire set of proteins encoded by a micro organism-the aims of the new field of proteomics. This information will be invaluable for understanding how complex biological processes occur at a molecular level, how they differ in various microorganisms, and how they are altered in different growing conditions as space conditions particularly in the case of the Melissa project. The spectrum of proteins expressed in a microorganism provides that cell with its unique identity. Elucidating how the protein complement changes in a cell type during development in response to

environmental stimuli as oxidative stress, temperature or pH variation. Recent years have witnessed a revolution in the development of new approaches for identifying large numbers of proteins expressed in cells and also for globally detecting the differences in levels of proteins in different cell states. In recent years, protein separation methods as the two-dimensional gel electrophoresis coupled with various mass spectrometry (MS) technologies have evolved as the dominant tools in the field of protein identification and protein complex deconvolution. This approach is probably the best approach to characterise the proteome especially if the genome is partial or unknown. Among the different steps of this approach, the protein extraction is crucial for the out come of this approach.

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

Moreover, the effectiveness of mechanical devices used to lysis a bacterium is clearly a function of the bacterial structure: some bacterial species are easily lysed whereas other are fairly resistance. The result of these observations is that bacteria can now be classified according to their degree resistance to mechanical lysis especially by sonication.

**Sensibility of the bacterial lysis by sonication**

***Rhodospirillum Rubrum>>>>>>Ralstonia metallidurans>>>>>Arthrospira platensis***

In conclusion, Proteomic approach based to 2-DE coupled to mass spectrometry has been shown to be a excellent means of obtaining information about physiological status and metabolism of the MELiSSA strains that is almost impossible to obtain in any other way, especially with an non or partially characterise genome. It makes it an ideal tool to understand in the molecular level the influence of various stresses (for example, X and UV-irradiations,

pressure, starvation, pH stress) on the strains present in the MELiSSA bioreactors. Finally, proteomic approach could be used to understand the behaviour of the MELiSSA bioreactors in different growth conditions

# 4 MALDI-TOF ANALYSIS OF THERMIC, OXIDATIVE AND PH STRESS ON *ARTHROSPIRA* SP. *PCC8005* AND *RHODOSPIRILLUM RUBRUM ATCC25903.*

## *4.1. Introduction*

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

A variety of characteristics of microorganisms (both phenotypic and genotypic) are used to differentiate between strains and often to determine the relationship between strains of bacteria present on the bacterial cell wall are surface components, which give rise to a unique pattern of biomarkers. These surface components are important since they mediate the contact between the cell and the environment. This unique population of molecules can be rapidly desorbed from the cell surface; ionised and analysed by matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF-MS) resulting in a mass fingerprint. This approach should be rapid and yet be based on a sufficiently large group of proteins so that a unique mass spectral fingerprint for the organism or strain can be obtained. Some of the difficulties involved in the MALDI-TOF mass spectral analysis of bacteria have been the complexity of the spectra, the large mass ranges used, and the subtle differences that may be observed in spectra from related stains. Moreover, reproducibility is a difficult problem in experiments that involve MALDI of cells, and large variations have been seen in spectra obtained under different conditions. There are many experimental parameters that can have a strong effect on the observed mas spectra (minor variations in the sample/matrix preparation, in the experimental conditions used to bacterial extraction or analysis). Here we report the success of the intact mass spectrometry to study statistically and with a high reproducibility *Rhodospirillum rubrum* ATCC25903 and *Arthrospira* sp. PCC8005. Another initially perplexing issue was the time dependence of spectra noted upon analysis of bacteria from cultures. Bacteria respond rapidly to environmental changes, and the production of stress proteins or other similar changes in cellular processes results to a modification in the spectra for reasons associated with the biology of bacteria. Our results demonstrated that MALDI-TOF of Whole cells could be used to monitor biological changes, such as those that occur during oxidatif , temperauture and pH stress. This whole-cell approach to the monitoring of biological changes would, of course, only allow a fraction of the environmental-response related proteins to be detected, compared to similar studies with isolated or fractionated protein samples, but the rapidity of the analysis often offsets this limitation.

## 4.2. Stress conditions

*Resistance to temperature stress:* A 100 µl aliquot of bacterial culture was transferred to a 1 ml tube (Eppendorf). For temperature stress, samples were stored at one of those temperatures for one hour. The temperature range was -170°C (liquid nitrogen), -80°C, -20°C, 4°C, 15°C, 28°C, 37°C, 45°C, 50°C, 60°C and 70°C. Controls were obtained by incubating bacteria at 28°C. After incubation, the tubes were centrifuged and the supernatant were discarded. Bacteria were then washed with 200µl of Water (mQ). After centrifugation, bacteria were resuspended in 10µl of water (mQ) and 1µl is spotted on the MALDI plate.

*Resistance to oxidative stress:* A 100 µl aliquot of bacterial culture was transferred to a 1 ml tube (Eppendorf). Oxidative stress was generated by addition of hydrogen peroxide (H2O2 30%, Merck) for an hour at the following final concentrations: 13,25; 27,5; 55; 110; 220; 440 and 880 mM. 10 µl of hydrogen peroxide (10 times more concentrated than the different concentrations tested) was added to the bacterial suspension and samples were stored at room temperature for an hour. Controls were obtained by incubating bacteria without any hydrogen peroxide. After incubation, the tubes were centrifuged and the supernatant were discarded. Bacteria were then washed with 200µl of Water (mQ). After centrifugation, bacteria were resuspended in 10µl of water (mQ) and 1µl is spotted on the MALDI plate.

*Resistance to pH stress:* A 100 µl aliquot of bacterial culture was transferred to a 1 ml tube (Eppendorf). Oxidative stress was generated by addition of sodium hydroxide or hydrogen chloride for an hour at the following final pH: 4, 5, 6, 7, 8, 9, 10 and 11. The final pH of the bacterial suspension is evaluated with a pH indicator strip. Bacterial suspension and samples were stored at room temperature for an hour. Controls were obtained by incubating bacteria in pH 7. After incubation, the tubes were centrifuged and the supernatant were discarded. Bacteria were then washed with 200µl of Water (mQ). After centrifugation, bacteria were resuspended in 10µl of water (mQ) and 1µl is spotted on the MALDI plate.

## 4.3. Mass spectrometry measurements

Mass spectrometry measurements were performed on a M@LDI LR (Micromass, Manchester, UK) laser desorption time-of-flight instrument equipped with a nitrogen UV laser ($\lambda$=337 nm). The laser fluency was set just above the threshold for ion production. The mass spectrometer was 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 was 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/ul, bovine insulin, 2pmol/ul and ubiquitin, 10pmol/ul). The acquisition mass range was from m/z 500 to 10000 Da. For maximum throughtput of samples the supernatant mass fingerprints were acquired automatically. Spectra from the reference wells, for lock mass calibration, were also acquired automatically. Twelve target wells were used for each organism to be entered into the database. A 1ul aliquot of matrix was applied to each target spot allowed to air-dry prior to

mass spectrometry analysis. Samples were mixed (1/1, v/v) with cyano-4-hydroxycin-namic acid on the target and dried in room temperature.

## *4.4. Data analysis*

Spectral data were 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 was determined by comparing each of the twelve replicate spectra to each other using the root mean square (RMS) value. An RMS rejection value of 3 used to identify significant outliers and an average mass spectral fingerprint obtained from a minimum of ten accepted replicate spectra of an individual bacterium was used to populate the database. The pattern recognition algorithm uses all the mass and intensity data in the mass spectrum to give the best database match with a probability score. A comparative display of the test spectrum and the differences from the best database match is produced in a browser format.

For temperature stress, bacteria incubated at 28°C were used as as positive controls for the intensity of the laser. For oxidative stress, bacteria incubated in the absence of hydrogen peroxide or at pH 7 were used as as positive controls for the intensity of the laser. Data (intensity of the different ions) were normalized and expressed as percentages of the value obtained for cells incubated at 28°C (for temperature stress), at pH 7.0 (for pH stress) or without any hydrogen peroxide (for oxidative stress) .

## *4.5. Results*

Figures 28 shows the effect of temperature stress on the fingerprint *R. rubrum* and *Arthrospira* sp.. Concerning *R. rubrum*, a significant decrease of pic surface of some ions essentially the ions m/z 9750 and 4875 could be observed when cells were submitted to low (-170°C, -80°C, -20°C) or high (+50°C, 60°C and 70°C) temperatures. Concerning *Arthrospira* sp., a relevant decrease of only one surface of ion of m/z: 861) could be noted when cells were submitted to 60 and 70 °C. The other ions do not show a statistically variation in function of the temperature as well for *R. rubrum* than for *Arthrospira* sp.. In conclusion, *R. rubrum* was more sensitive to temperature variation in comparison with *Arthrospira* sp.. Each bacterial strain showed characteristic ions that were attributed to bacterial membrane and intracellular proteins and metabolites. It is know that high-mass ions as it observed to *R. rubrum* (ions m/z 9750 and 4875) were attributed to intracellular bacterial proteins. The ion m/z 4875 was characterised by a double charge and corresponds to the same protein that the ion m/z 9750. For *R. rubrum*, the nature of the protein characterised by a m/z 9750 is unknown but its decrease in high and low temperature could correspond to a partial lysis of the bacteria due to a increase of the membrane permeability in function of the temperature.

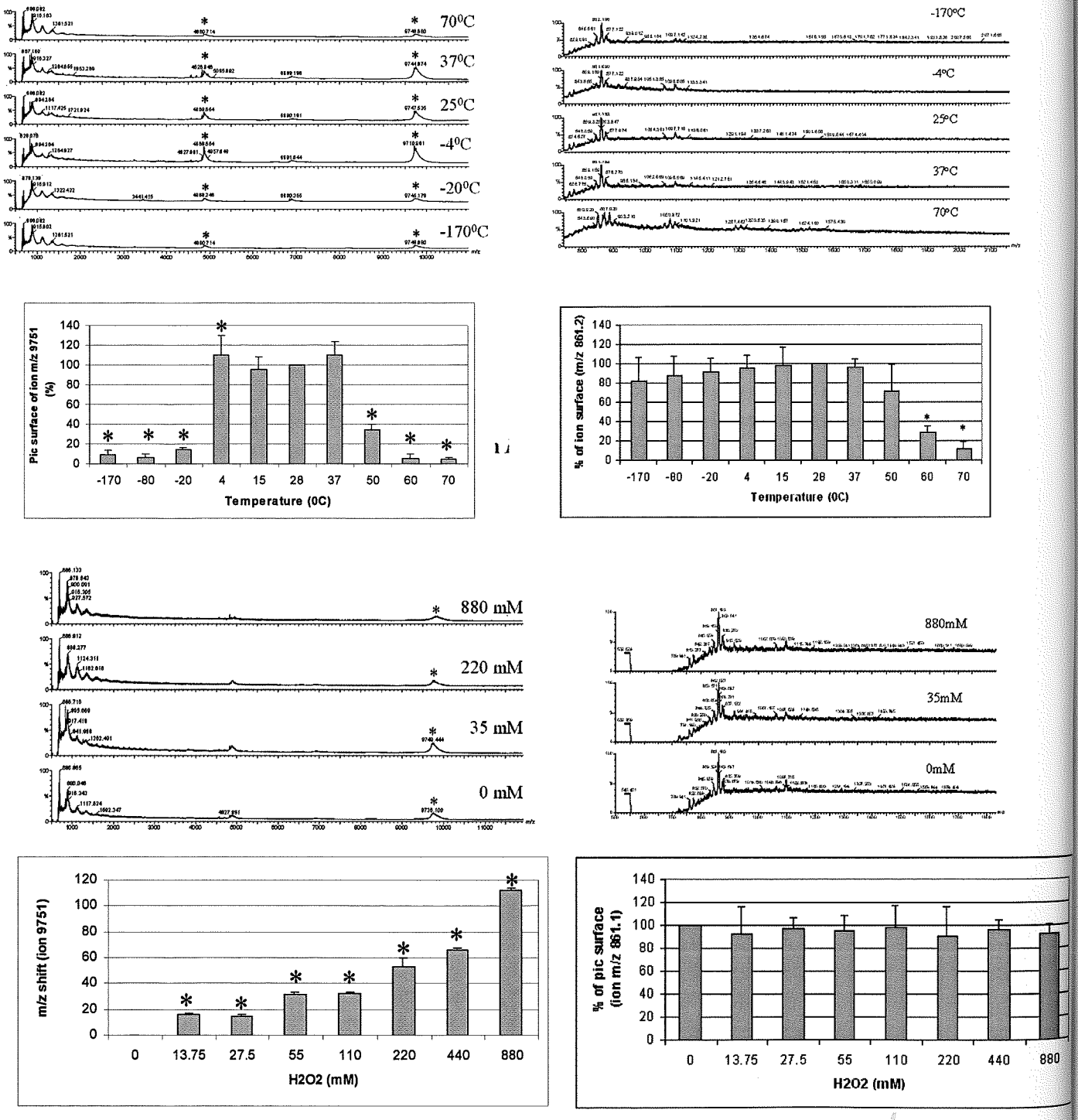



**Figure 4.2. Effect of oxidative stress on *R. rubrum (left)* and *Arthrospira* sp. (right).**

The loss of membrane integrity represents significant damage for cells due to multiple functions linked to the plasma membrane (permeability barrier, transport, respiratory activity, etc..). Our results showed different ion variations in the specific spectra of the both bacteria that reveal a difference in the resistance of the strains to temperature stress. *R. rubrum* seemed to show higher sensitivity to temperature stress than *Arthrospira sp.*

Figures 4.2. shows the effect of oxidative stress on fingerprint of *R. rubrum* and *Arthrospira* sp.. After oxidative stress, a mass shift of some ions (in comparison with control conditions) in function of the concentration of $H_2O_2$ was observed in *R. rubrum* while no significant change was observed in the spectra of *Arthrospira* sp.. The mass shift observed, i.e for the ions m/z 9750 and 4875, correspond to an increase of the oxidation state of this corresponding protein (multiple of 16 Da). Interestingly, Oxidative stress, measured by flow cytometry, induced an increase in membrane permeability that was, in contrast for the *Arthrospira* sp., clearly proportional to the concentration of $H_2O_2$ in *R. rubrum.*

Figure 4.3. shows the effect of pH stress on fingerprint of *R. rubrum*. After pH stress, a significant decrease of pic surface of some ions essentially the ions m/z 9750 and 4875 was observed when cells were submitted to low pH 6,5,4 and 2) or high (pH 8,9,10,11 and 12) pH. The other ions do not show a statistically variation. Concerning *Arthrospira* sp., the analyses still have to be done. In conclusion, the decrease of the surface pic corresponding to the ions m/z 970 in high and low pH could correspond to a partial lysis of the bacteria due to an increase of the membrane permeability as observed during temperature stress. Interestingly, a significant increase of membrane permeability analysed by flow cytometry was observed when cells were submitted to the same low (pH 6,5,4 and 2) or high (pH 8,9,10 and 12) pH.

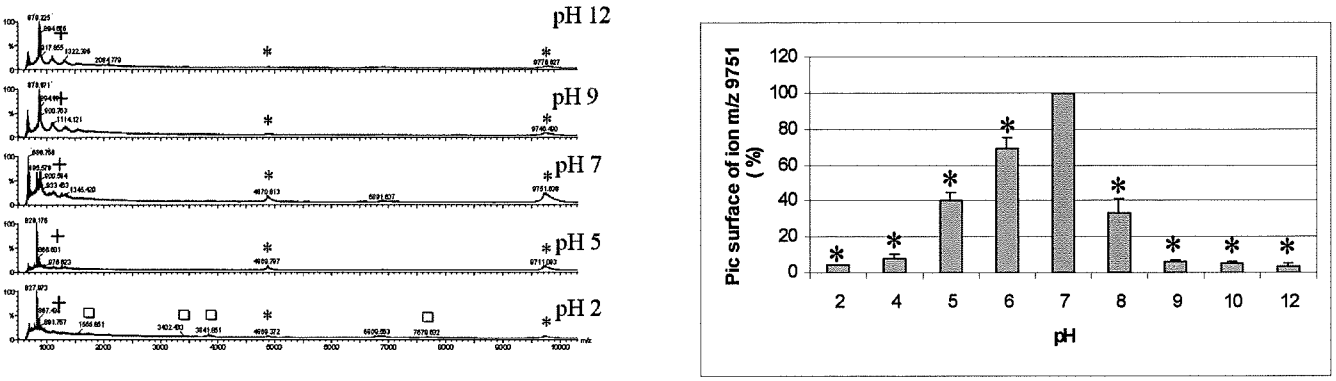



**Figure 4.3. Effect of pH stress on *R. rubrum* .**

## *4.6. Conclusion*

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

In this work, we were interested in the application of mass spectrometry to monitor the effect of different stresses that can happen in space (temperature, oxidative and pH stress) on some of the MELiSSA bacterial strains. Bacteria respond rapidly to environmental changes, and the production of stress proteins or other similar changes in cellular processes results to a modification in the mass spectra for reasons associated with the biology of bacteria. Our results demonstrated that MALDI-TOF of Whole cells could be used to monitor biological changes, such as those that occur during oxidatif , temperauture and pH stress. Usually, bacteria were sampled from colonies on an agar plate, mixed with the matrix, air–dried, and introduced in batches into the mass spectrometer for analysis. In our work, mass spectra were directly obtained from liquid culture. Experimental conditions have been establish to obtain specific and reproducible spectra from the different MELiSSA strains. Moreover, our results indicate that the *R. rubrum* and *Arthrospira* sp. strains showed different behaviours to oxidative, pH and temperature stresses. *R. rubrum* seemed to show higher sensitivity to temperature, pH and oxidative stresses than *Arthrospira* sp. Interestingly, These results were in good concordance with those obtained by flow cytometry. Moreover, in both strains, a correlation was observed between membrane integrity and the mass spectrum modifications observed during stress variations.

Mass spectrometry especially applied on the whole cells has been shown to be a fast, excellent means of monitoring the effect of different environmental stresses on the strains present in the Melissa bioreactors. Nevertheless, this approach is not able, without a prefractionation, to monitor specific physiological modifications from heterogeneous populations of a bacteria strain, as *Arthrospira platensis* constituted of small and big filaments. Finally, mass spectrometry could be also used to identify intact cyanobacteria and to detect their secondary toxic metabolites.

In another interesting way, pigments and proteins from chlorosomes, the light-harvesting organelles from the photosynthetic bacterium could be characterized directly from organelles. Recent work shown that by applying a small volume of a concentrated suspension of isolated chlorosome organelles directly onto the MALDI target, bacteriochlorophyll A and the major homologs of bacteriochlorophyll C have been characterised from *Chlorobium tepidum.* Interestingly, the authors noted that the peak surface of the different bacteriochlorophyl in the MALDI spectra were proportional to peak areas obtained by HPLC analysis. Similar results

were reported when whole cells were applied to the target. The MALDI-TOF can rapidly provide semiquantitative analysis as well as a fingerprint for the small amount pigments present in bacteria. This approach could be applied to the MELiSSA strains to monitor their light-harvesting systems in different growth conditions.

# 5 OPTIMISATION OF MOLECULAR METHODS FOR GENOMIC ANALYSIS OF *ARTHROSPIRA* SP. PCC8005

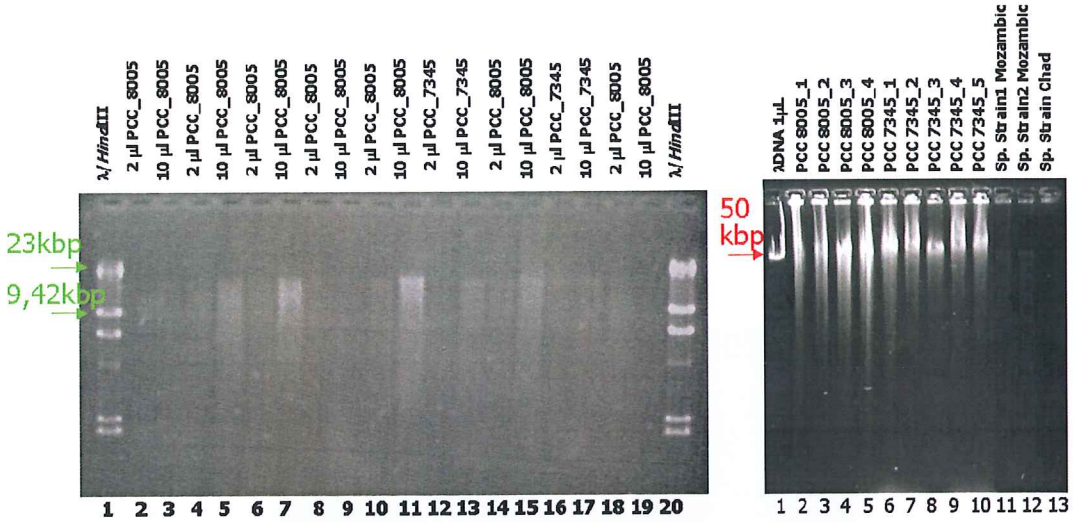
## *5.1 Introduction*

One method that has been selected for the detection of genomic changes in *Arthrospira* in response to stresses is Amplified Fragment Length Polymorphism (AFLP). However, during preliminary trials on the MELiSSA strains, Dr Janssen observed that it was very difficult to obtain good quality DNA from *Arthrospira* sp. PCC8005 and to perform AFLP, whereas he had more success with *R. rubrum* ATCC25903 and *R. metallidurans.* Therefore, some effort had to be dedicated to find a suitable method for DNA extraction from *Arthrospira.* A second step towards the AFLP was carried out by testing several restriction enzymes for digestion of the genomic DNA.

We also have studied the affiliation of the *Arthrospira* strain that is currently being sequenced at the Human Genome Center in Peking. We have obtained 3 subcultures of the strain 'China', that have been already purified by a student of Prof. Zhang. We have determined in which cluster they belonged, on the basis of the ITS (Internally Transcribed Spacer) between the 16S and 23S rRNA genes.

## *5.2 Extraction of genomic DNA from Arthrospira strains.*

The total genomic DNA was extracted from the *Arthrospira* strains using 3 different kits ( NucleoSpin Plant Kit (Macherey-Nagel), Wizard DNA Kit (Promega), DNeasy Plant Mini Kit (Qiagen)) and a classical method using phenol/chloroform and Proteinase K (Ausubel et al. 1992) with the following modifications. Centrifuged cyanobacterial cells were washed 3x in hot (80°C) TE buffer (50mM Tris, 40 mM EDTA). This buffer, with an increased concentration of the salts, inhibits the non-specific nucleases after lysis and removes polysaccharides. Then, the cells pellets were frozen at -20°C. Freezing the cells increases the efficiency of lysis. However, the cells cannot be stored for a long period. Then, an incubation with Proteinase K was performed at 60°C instead of 37°C. At this temperature, Proteinase K is most active and some of the enzymes are faster inactivated (e.g. restriction enzymes and mesophilic and psychrophilic nucleases). In addition, the lysis at 60°C is more efficient. After the precipitation step, the DNA was resuspended for minimum 3 days at 4 °C. The DNA is stored at 4 °C to avoid damages inflicted during freezing and thawing. The comparison of the quality of the DNA obtained with kits and a classical method shows that kits yielded only small fragments of DNA (< 20 Kbp) and that the DNA was of bad quality. However, the DNA extracted with a classical method had a good quality and showed fragments sizes of about 50 Kbp (Fig. 1). In addition, it was not degraded even after 16h incubation at 37 °C (Fig. 2).

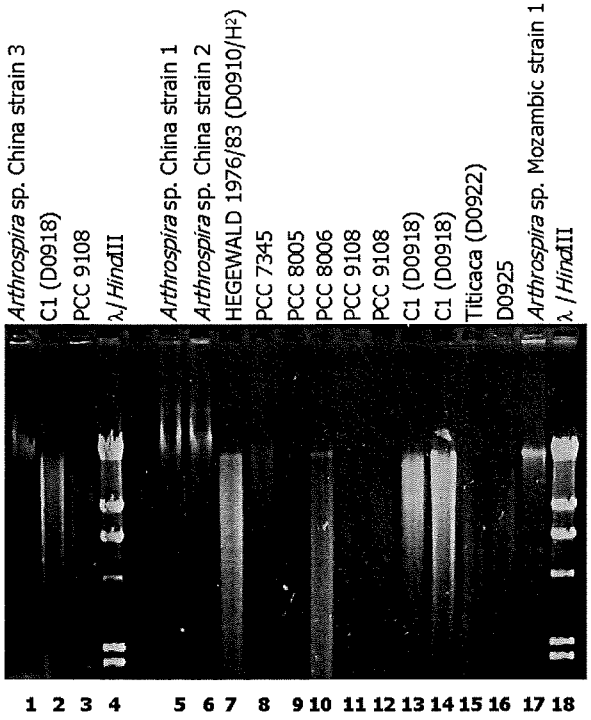



**Panel A**
**Lanes 1, 20 - DNA Ladder λ /*Hind*III**
**Lanes 2-7 - DNA extracted from *Arthrospira* strain PCC 8005 with the NucleoSpin Plant Kit. There were two modifications: addition of Proteinase K during lysis (lanes 4, 5), and boiling of the cells during the lysis (lanes 6, 7).**
**Lanes 8, 9 - DNA extracted from *Arthrospira* strain PCC 8005 with the Wizard DNA Kit.**
**Lanes 10, 11 - DNA extracted from *Arthrospira* strain PCC 8005 with the Wizard DNA Kit with boiling of the cells during the lysis.**
**Lanes 12, 13, 16, 17 - DNA extracted from *Arthrospira* strain PCC 7345 with the DNeasy Plant Mini Kit.**
**Lanes 14, 15, 18, 19 - DNA extracted from *Arthrospira* strain PCC 8005 with the DNeasy Plant Mini Kit.**

**Panel B**
**Lane 1 – Undigested λ DNA.**
**Lanes 2 - 5 - DNA extracted from *Arthrospira* strain PCC 8005 with a classical method with modifications.**
**Lanes 6 - 10 DNA extracted from *Arthrospira* strain PCC 7345 with a classical method with modifications.**
**Lane 11 - DNA extracted from *Arthrospira* strain 1 from Mozambique**
**Lane 12 - DNA extracted from *Arthrospira* strain 2 from Mozambique**
**Lane 13 - DNA extracted from *Arthrospira* strain from Chad.**
**(Lanes 2-10 - DNA was extracted from fresh cultures which were washed with TE buffer and frozen at - 20°C. Lanes 11-13 – DNA was extracted from fresh cultures that were not frozen.)**

**Fig. 1. DNA extraction with different methods: NucleoSpin Plant Kit (Macherey-Nagel), Wizard DNA Kit (Promega), DNeasy Plant Mini Kit (Qiagen) (Panel A), and with a classical method with modifications (Panel B).**

For DNA extraction, fresh and frozen (-20 °C) cells of *Arthrospira* were used. It was observed that the DNA was more degraded when the *Arthrospira* cells had been kept several years at -20 °C (Fig. 3). For extraction, fresh cultures should be used, or the cells should be stored at -80 °C.




**Lanes 4 and 18 – DNA Ladder λ /*Hind*III.**
**Lanes 1, 5, 6 – DNA extracted from fresh cultures frozen for several days.**
**Lanes 2, 3, 7 -17– DNA extracted from cultures frozen since 5 years.**

**Fig. 3. DNA extraction by a classical method (with phenol/chloroform and Proteinase K) with modifications from cultures frozen for several days and from cultures frozen since 5 years.**

Also, it was observed that is difficult to obtain the same quantity and quality of DNA even using the same method. A good quality DNA should be aliquoted, kept at -80 °C (avoiding refreezing) and used during the whole project to avoid a possible unreproducibility of the results of AFLP.

## *5.3 Start-up of AFLP*

Digestions of chromosomal DNA from 2 *Arthrospira* strains (PCC 8005 and PCC 7345) with 4 restriction enzymes: *Mse*I, *Hind*III, *ApaI, XhoI* were performed to check the usefulness of the endonucleases chosen for AFLP. The results of these experiments indicated that *Mse*I cuts very often and efficiently the DNA of tested *Arthrospira* strains (Fig. 3), *ApaI* and *Xho*I cut

rarely (Fig. 4), whereas *Hind*III does not cut or cuts not efficiently the DNA of the *Arthrospira* strains PCC 8005 and PCC 7345 (Figs. 3, 4).

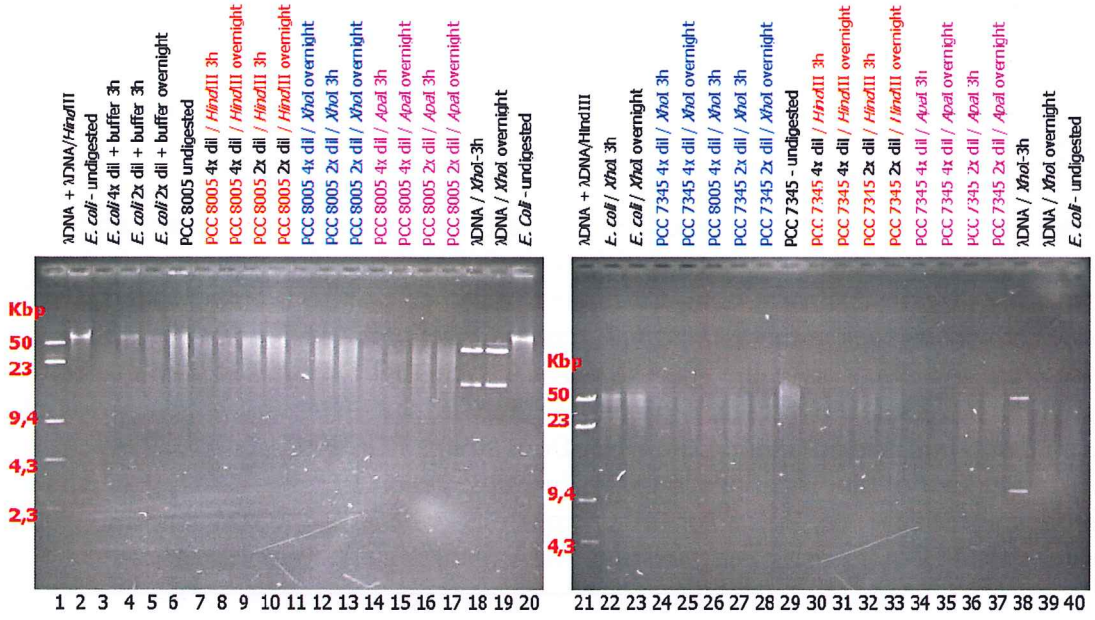



**Fig. 4. Digestion of diluted (4x and 2x) chromosomal DNA from *Arthrospira* strains PCC 8005 and PCC 7345, with *ApaI, XhoI* and *Hind*III.**

**Lanes 1, 21 - Undigested λ DNA + λ DNA digested with *Hind*III.**
**Lanes 2, 20, 40 - Undigested chromosomal DNA from *E. coli***
**Lanes 3, 4, 5 – 2 or 4 times diluted chromosomal DNA from *E. coli* incubated with buffer for restriction endonucleases at 37 °C for 3h and over the night.**
**Lane 6 - Undigested DNA extracted from *Arthrospira* strain PCC 8005.**
**Lanes 7, 8, 9, 10 – Digestion of 2 and 4x diluted chromosomal DNA from *Arthrospira* strain PCC 8005 with *HindIII* enzyme at 37 °C for 3h (lanes 7, 9) and overnight (lanes 8, 10).**
**Lanes 11, 12, 13 - Digestion of 2 and 4x diluted chromosomal DNA from Arthrospira strain PCC 8005 with XhoI at 37 °C for 3h (lane 12), and overnight (lane 11, 13).**

**Lanes 14, 15, 16, 17 - Digestion of 2 and 4x diluted chromosomal DNA from Arthrospira strain PCC 8005 with ApaI at 37 °C for 3h (lanes 14, 16), and overnight (lanes 13, 15).**
**Lanes 18,19, 38, 39 - Control of the enzymes *XhoI* and *ApaI* activity: λ DNA digested with XhoI at 37 °C for 3h (lane 18), and overnight (lane 19), λ DNA digested with ApaI at 37 °C for 3h (lane 38) and overnight (lane 39).**
**Lane 22, 23 – Chromosomal DNA from *E. coli* digested with XhoI at 37 °C for 3h (lane 22), and overnight (lane 23).**
**Lanes 24, 25, 27, 28 - Digestion of 2 and 4x diluted chromosomal DNA from Arthrospira strain PCC 7345 with XhoI at 37 °C for 3h (lanes 24, 26), and overnight (lane 25, 27).**
**Lane 26 - Digestion of 4x diluted chromosomal DNA from *Arthrospira* strain PCC 8005 with *XhoI* enzyme at 37 °C for 3h.**
**Lanes 29 - Undigested DNA extracted from Arthrospira strain PCC 7345.**
**Lanes 30, 31, 32, 33 - Digestion of 2 and 4x diluted chromosomal DNA from Arthrospira strain PCC 7345 with HindIII at 37 °C for 3h (lanes 30, 32), and overnight (lane 31, 33).**
**Lanes 34, 35, 36, 37 - Digestion of 2 and 4x diluted chromosomal DNA from *Arthrospira* strain PCC 7345 with ApaI at 37 °C for 3h (lanes 34, 36), and overnight (lane 35, 37).**

In conclusion, for AFLP, we will be able to use *Mse*I and *Xho*I or *Apa*I.
Prof. Cheng-Cai Zhang, who is involved in the sequencing of one *Arthrospira* genome in China, informed us that the sites of the restriction enzymes *Hind*III, *Xho*I or *Apa*I occur rarely in the genome of their *Arthrospira* strain. The average lengths of the fragments produced by these enzymes were 15.5 kbp, 30 kbp and 48 kbp, respectively. Prof. Cheng-Cai Zhang suggested to use different enzymes, which cut the *Arthrospira* genome more frequently: *Bam*HI (10.18 kbp), *Eco*RI (7.37 kbp), *Bsa*BI (3.18 kbp), *Eco*RV (2.44 kbp), *Cla*I (4.24 kbp), *Hinc*II (1.24 kbp).

We are specially interested in the endonuclease *Eco*RI because it has been used for AFLP to differentiate *Nostoc linckia* strains (Satish et al., 2001). Thus, the primers and adapters needed are already described. Prof. Cheng-Cai Zhang informed us also that there are several possible DNA methylase genes in the *Arthrospira* genome, e.g.: type I restriction-modification systems, DNA methylases, cytosine-specific DNA methyltransferases, site-specific DNA methyltransferases. The presence of methylation in the *Arthrospira* genome could be the reason that *Hind*III is not cutting the DNA from strains PCC8005 and PCC7345. The presence of different kind of modifications in the *Arthrospira* genome (methylation, glycosilation) could complicate the use of AFLP or other methods which are based on the restriction of chromosomal DNA from *Arthrospira* (e.g. RDA method).

## 5.4 Conclusions

We have set up a DNA extraction method allowing to obtain High Molecular Weight DNA, what is necessary for AFLP and other techniques.

Several tests of restriction enzymes for the digest was carried out and showed that *MseI*, *Xho*I and *Apa*I managed to cut the DNA of strain PCC8005. However, we have got later the results from a 'in silico' analysis of the contigs obtained for the sequenced strain in China and on this basis, *Eco*RI could be chosen instead of *Xho*I and *Apa*I, because it cuts the genome more frequently.

## 5.5 References

Ausubel, F. Brent, R., Kingston, RE., Moore D., Seidman, JG., Smith JA., Struhl, K (1999) Short protocols in Molecular Biology, 1104 pp., Wiley & Sons, USA.

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Scheldeman, P., Baurain, D, Bouhy, R., Scott, M., Mühling, M.,Whitton, B.A., Belay, A. & A. Wilmotte (1999) *Arthrospira* ('*Spirulina*') strains from four continents are resolved into only two clusters, based on amplified ribosomal DNA restriction analysis of the internally transcribed spacer. FEMS Microbiol. Lett. 172: 213-222.

Satish N, Krugman T, Vinogradova ON, Nevo E, Kashi Y. (2001) Genome Evolution of the Cyanobacterium *Nostoc linckia* under Sharp Microclimatic Divergence at "Evolution Canyon," Israel. Microb Ecol. 42:306-316.

# 6 MORPHOLOGICAL AND GENETIC DIFFERENTIATION OF *ARTHROSPIRA* STRAINS

## *6.1 Introduction*

We needed to know if the strain presently sequenced in China was closely related or identical to the MELiSSA strain PCC8005. However, the origin of the strain was lost. Moreover, it was also hypothesized that the sequencing problems encountered in Peking could be due to the presence of contaminants. Thus, we have received 3 subcultures of the China strain thanks to Prof. CC Zhang and we have carried out a genotypic characterisation based on the ITS sequence. It appeared that the 3 subcultures are identical and belong to the ITS-cluster II.A. As we have already characterised 51 strains of *Arthrospira* from 4 continents with the same molecular marker (Scheldeman et al., 1999; Baurain et al., 2002), we have noted that the most likely candidate, as origin of the China strain, was PCC9108.

## *6.2 Morphological identification, and study of the purity, of the three unknown Arthrospira subcultures from China.*

The microscopic observation of 5 *Arthrospira* strains: PCC 8005, PCC 7345 and 3 China isolates, revealed that the China subcultures seemed to be pure, without any bacterial and fungal contamination, and they were more similar (positions of gas vesicles) to the type strain PCC 7345 than to the strain PCC 8005. However, when plated on solid LB medium, bacterial growth was visible in the China strain.

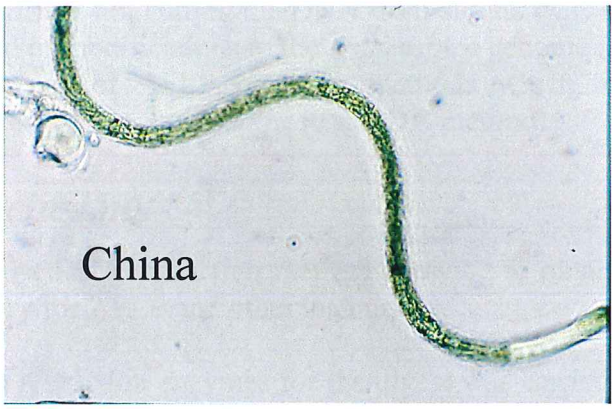



**Fig. 6.1. China strain 1, with gas vesicles dispersed into the cell**

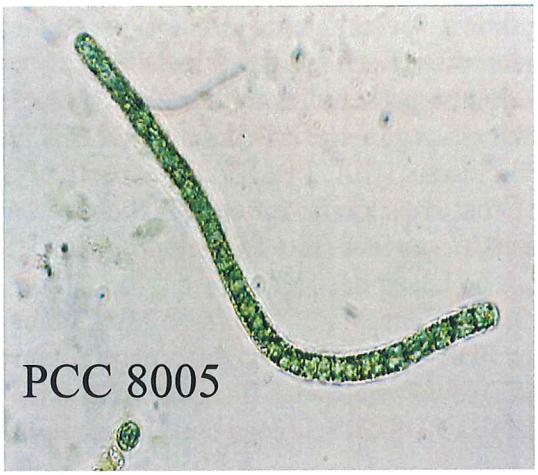



**Fig. 6.2. Strain PCC 8005, with gas vesicles concentrated at the cross-walls**

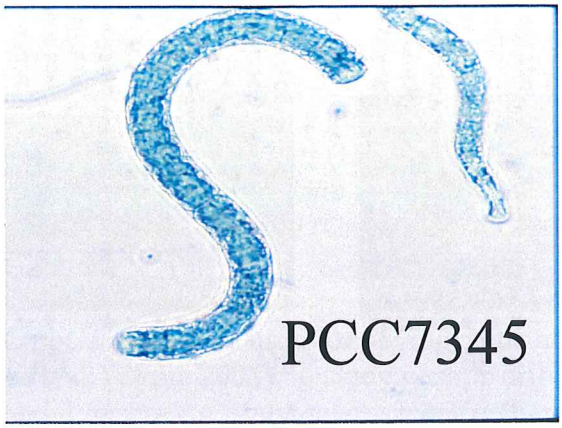



**Fig. 6.3. Strain PCC7345, with gas vesicles dispersed into the cell**

## *6.3 Genotypic differentation between Arthrospira sp. strains*

To identify the China strains, the 16S and ITS region were sequenced for these three isolates, using PCR primers specific for cyanobacteria. The analysis of the obtained sequences revealed that ITS sequences of the China strains were identical and belonged to the ITS cluster II.A (containing a.o. strain PCC 9108 from China), whereas there was only 97% of similarity between the China strains and our MELISSA strain PCC 8005. Our 'educated guess' is that strain PCC9108 is the most likely candidate to be the ancestor of the strain that is currently sequenced in Peking.

Concerning the history of strain PCC9108, it was isolated by R. Lewin as *Spirulina platensis*, in a commercial culture facility of Cheng-hai, Yunnan, China, and was deposited in the Pasteur Culture Collection of Paris. In addition, several characteristics ( W/D 8 - 10 µm; motility permanent; gas vacuoles permanent, dispersed) indicated the similarity of this strain to the China isolates, e.g. the same position of gas vesicles in the cells.
From previous studies of the *Arthrospira* genome it is known that PCC 9108, PCC 8005 and PCC 7345 belong to different ITS clusters (II.A, I.B, II.A/B respectively). The primers designed by Baurain et al. (2002), that are specific for each ITS cluster, allowed to affiliate the China strains of *Arthrospira* to the Cluster II.A (like PCC 9108) (Fig. 5).

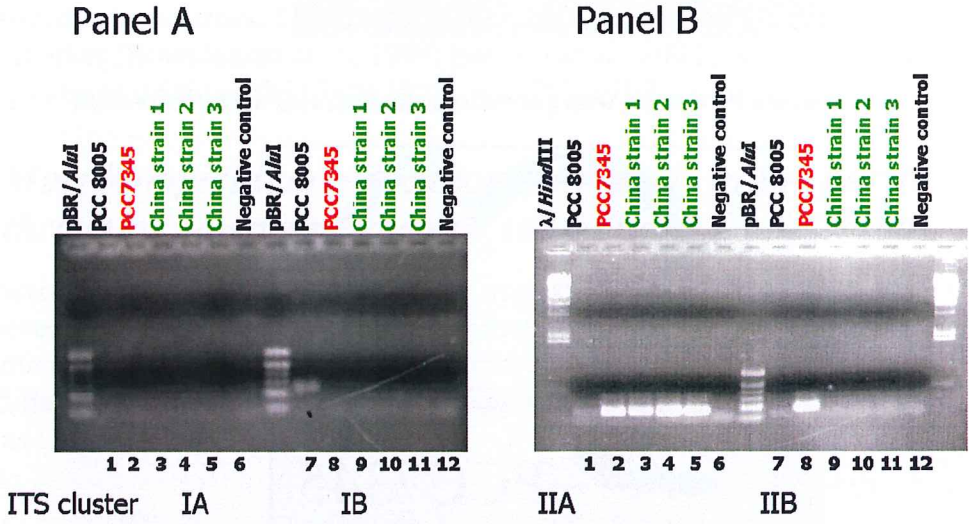



**Fig. 5. Amplification of the ITS region from *Arthrospira* strains PCC 8005 and PCC7345 and from China isolates with specific primers.**

**Panel A : Amplification of the ITS region from *Arthrospira* strains PCC 8005 and PCC7345 and from China isolates with primers specific for ITS subcluster I. A (lanes 1-6) and primers specific for ITS subcluster I. B (lanes 7-12).**
**Panel B : Amplification of the ITS region from *Arthrospira* strains PCC 8005 and PCC7345 and from China isolates with primers specific for ITS subcluster II. A (lanes 1-6) and primers specific for ITS subcluster II. B (lanes 7-12).**

Using techniques described in the following section, the similarity of China strains and strain PCC 9108 was confirmed by sequences of other loci, *recA* and *gyrA*.

The genus *Arthrospira* consists of a dozen species (Jeeji-Bai, 1999) that are difficult to distinguish morphologically. A molecular characterisation based on the ITS (Internally Transcribed Spacer) of the rRNA operon from 50 *Arthrospira* strains revealed that there were 4 genotypes grouped into 2 clusters (I and II). Inside each of them, 2 subclusters were observed (I.A, I.B and II.A, II.B). The 5$^{th}$ genotype was a consequence of the presence of two ITS forms II.A and II.B in the copies of the same organism (Scheldeman *et al.* 1999; Baurain *et al.* 2002).

To study the genetic variability of several *Arthrospira* strains belonging to different ITS clusters, we have used a polyphasic approach based on the polymorphism of 4 DNA loci: *gyrA, recA, rpoC* and *cpcBA* operon. The presence of highly conserved motifs in genes encoding these proteins enable the construction of PCR primers suitable for the differentiation of bacteria at the genera, species and subspecies level (Huang, 1996). Moreover, 2 fingerprinting methods have been used: ERIC-PCR (Versalovic *et al.* 1991) and HIP-PCR (Bhaya *et al.*, 2000).

RecA protein and *recA* gene sequence comparisons have been used to study the phylogenetic relationships among bacterial genera and species (Lloyd & Sharp, 1993). The *recA* gene has been used for typing of acinetobacteria (Nowak & Kur, 1995) for example.

Studies on the polymorphism of the *gyrA* gene have been used for the genotyping of the following bacteria species: *Pseudomonas* (Hocquet *et al.*, 2003), *Mycobacterium tuberculosis* (Fletcher *et al.*, 2003), *Campylobacter jejuni* (Ragimbeau *et. al.*1998), *Neisseria gonorrhoeae* (Deguchi *et. al.* 1998), *Salmonella typhi* (Calva *et. al.* 1997, Arya 1998), *Bartonella sp.* (Sander *et. al.* 1998).

The sequences of *rpoC1* gene were used for phylogenetic analysis of 5 subgroups of cyanobacteria (Seo and Yokota 2003), to study genetic differentiation of strains belonging into several cyanobacterial genera e.g. *Anabaena circinalis* (Fergusson and Saint, 2000), *Cylindrospermopsis raciborskii* (Wilson et al., 2000), *Synechococcus sp.* (Toledo and Palenik,1997, Toledo et al. 1999).

In addition, sequences of *cpcBA* operon have been used to study the phylogenetic diversity of cyanobacteria among freshwater picocyanobacteria and toxic cyanobacteria (Crosbie et al. 2003, Robertson et al. 2001, Baker et al. 2001, Neilan et al. 1995) and to study the relationships inside the *Arthrospira* genus (Manen and Falquet, 2002).

New pairs of PCR primers were designed for the amplification of 3 housekeeping genes: *gyrA, recA* and *rpoC1*. They allowed us to amplify and sequence a fragment of each gene. For amplification of the *cpcB-A* locus primers Pc$\beta$F (Neilan *et al.*, 1995) and Pc*a*R (Manen and Falquet, 2002) were used. A comparison of the resolution power of the different DNA loci with the data of the ITS sequences was carried out.

## *6.4 Results*

### 6.4.1 POLYMORPHISM OF *RECA* GENE.

The 782 bp fragment of 8 *recA* genes (Fig. 1) from *Arthrospira* strains belonging to different ITS clusters (Table 1), were amplified and sequenced.

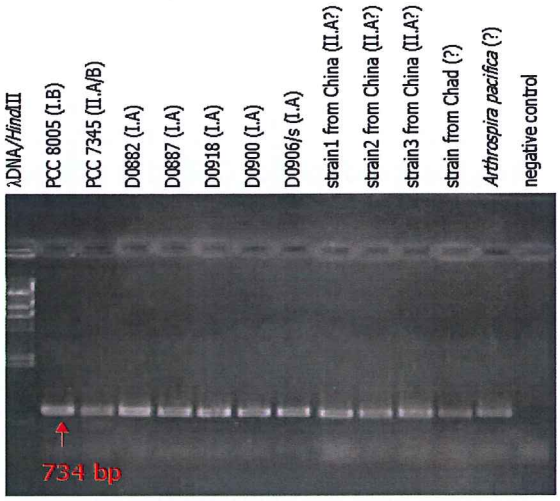



**Fig. 1. Amplification of the 782 bp fragment of the *recA* gene.**

The analysis of the 717 bp (out of 782 bp) fragment of *recA* gene sequences, revealed that there were 35 polymorphic positions (Fig. 2).

| | I.A | I.B | II.A | II.B | II.A/B PCC7345 | II.A/B D0881 |
|---|---|---|---|---|---|---|
| **I.A** | - | 0 | 31 | 35 | 30 | 35 |
| **I.B** | | - | 31 | 35 | 30 | 35 |
| **II.A** | | | - | 8 | 1 | 8 |
| **II.B** | | | | - | 7 | 0 |
| **II.A/B PCC 7345** | | | | | - | 7 |
| **II.A/B D0881** | | | | | | - |

**Fig. 2. Pairwise comparison of a 717 bp fragment from the *recA* sequences in relation to the ITS clusters. Numbers indicate the sequence differences between *Arthrospira* strains belonging to different ITS clusters.**

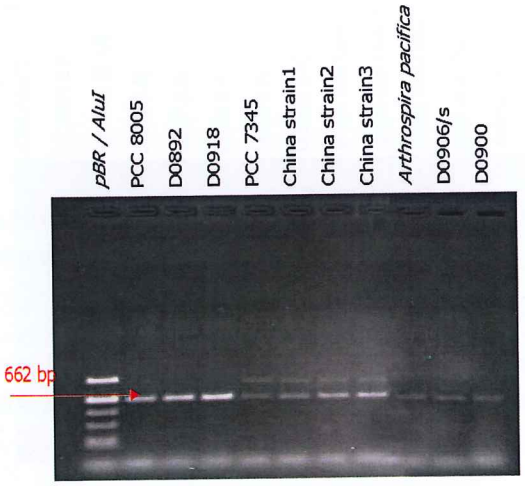
The *recA* sequences from *Arthrospira* strains belonging to ITS clusters I.A and I.B, which have only 2 different nucleotides in their ITS sequences, were identical. However, there were clear differences between strains belonging to clusters I and II (Fig. 2).

On the other hand, the *recA* sequences allowed to differentiate the strains belonging to cluster II.A and II.B (Fig. 2, – green triangle). Two strains, PCC 7345 and D0881 which belong to cluster II.A/B (Baurain et al. 2002) have different *recA* sequences (Fig. 2 - orange stars). Strain PCC7345 has a *recA* sequence that is almost identical to strains from cluster II.A. (Fig. 2 – blue squares), while strain D0881 was very similar to strains from ITS cluster II.B (Fig. 2 – red circle).

In the case of strains from clusters II.A, II. B and II.A/B, the *recA* sequences have a higher resolution power than ITS sequences because they show 7 or 8 polymorphisms instead of only 4 base differences. Interestingly, the translation of obtained *recA* sequences show that the amino acid sequences of RecA are identical for the cluster I and II strains. This illustrates the conserved nature of RecA protein.

## 6.4.2 POLYMORPHISM OF *GYRA GENE*.

The 662 bp fragment of *gyrA* gene (Fig. 3) from 12 *Arthrospira* strains belonging to different ITS clusters (Table 1), were amplified and sequenced. The 621 bp (out of 662 bp) *gyrA* gene fragment had 20 polymorphic sides (Fig. 4).



| | I.A | I.B | II.A | II.B | II.A/B PCC7345 | II.A/B D0881 |
|---|---|---|---|---|---|---|
| **I.A** | - | 0 | 20 | 19 | 20 | 19 |
| **I.B** | | - | 20 | 19 | 20 | 19 |
| **II.A** | | | - | 0 | 0 | 1 |
| **II.B** | | | | - | 1 | 0 |
| **II.A/B PCC 7345** | | | | | - | 0 |
| **II.A/B D0881** | | | | | | - |

**Fig. 4. Pairwise comparison of a 623 bp fragment of *gyrA* gene sequences in relation to the ITS clusters. Numbers indicate the sequence differences between *Arthrospira* strains belonging to different ITS clusters.**

In this case, differences were only found between strains belonging to clusters I and II. The *gyrA* sequences from *Arthrospira* strains belonging to ITS clusters I.A and I.B, were identical. Also, no differences were found between strains belonging to clusters II.A, II.B and II.A/B (Fig. 4). Strain D0881 which belongs to cluster II.A/B. (Baurain et al. 2002) has only one nucleotide difference with strains from cluster II.A (Fig. 4 – red circle) and strain PCC7345 differs at one position from the *recA* sequences of strains from cluster II.B (Fig. 4 – blue squares). Translation of the *gyrA* gene into aminoacids revealed that 2 aminoacids are different between strains belonging to ITS clusters I and II.

### 6.4.3 POLYMORPHISM OF *RPOC1* GENE.

The 734 bp fragment of *rpoC1* gene from 13 different *Arthrospira* strains (Table 1) belonging to different ITS clusters, were amplified and sequenced.

The analysis of the 704 bp (out of 734 bp) fragment of *rpoC1* gene sequences, revealed that there were 35 polymorphic positions (Fig. 5). The *Arthrospira* strains belonging to ITS clusters I.A and I.B show only 1 nucleotide difference in *rpoC1* gene sequences (Fig. 5). Furthermore, strains from cluster I are very similar to strains belonging to subcluster II.A. Strains belonging to cluster I and subcluster II.A are clearly different (about 30 bp) from strains belonging to

clusters II.B and II.A/B. Two strains, PCC 7345 and D0881, which belong to cluster II.A/B. have one nucleotide difference in their *rpoC1*sequences (Fig. 5) and both of them are similar to strains from ITS cluster II.B (Fig. 5. – red circles).

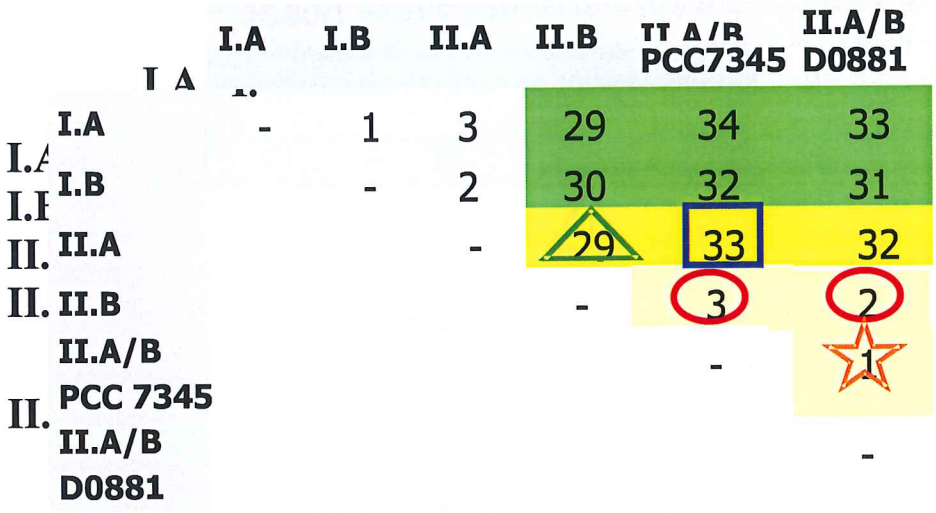



**Fig. 5. Pairwise comparison of a 704 bp fragment of *rpoC1* sequences in relation to ITS clusters. Numbers indicate the sequence differences between *Arthrospira* strains belonging to different ITS clusters.**

Interestingly, the translation of obtained *rpoC1* sequences show that the aminoacid sequences of *Rpo*C1 are identical for all *Arthrospira* strains. This illustrates the conserved nature of this protein.

## 6.4.4 POLYMORPHISM OF *CPCBA* OPERON.

The 668 bp fragment of the *cpcBA* locus from 18 different *Arthrospira* strains (Table 1) belonging to different ITS clusters, was amplified and sequenced. The particularity of this molecular marker is that it consists in partial sequences from 2 genes (*cpcB*, *cpcA*) and the spacer between them. The analysis of obtained sequences, revealed that there were 25 polymorphic positions in total (Fig. 6).

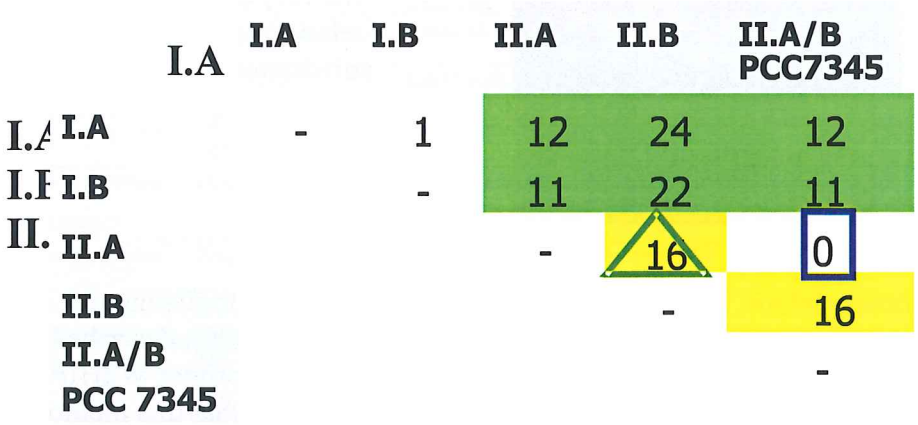



**Fig. 6. Pairwise comparison of a 621 bp fragment of *cpcBA* locus sequences, in relation to ITS clusters. Numbers indicate the sequence differences between *Arthrospira* strains belonging to different ITS clusters.**

The *cpcBA* locus sequences from *Arthrospira* strains belonging to ITS clusters I.A and I.B have only 1 different nucleotide (Fig. 6). There were clear differences between strains belonging to clusters I and II, and strains belonging to subclusters II.A and II.B (Fig. 6 – green triangle). Strain PCC7345 has a *cpcBA* locus sequence that is identical to strains from cluster II.A. (Fig. 6 – blue squares). Unfortunately we do not have the *cpcBA* locus sequences for strain D0881. The translation of the *cpcB* and *cpcA* genes into aminoacid sequences revealed that 3 aminoacids were different between strains belonging to ITS clusters I and II. Two of them were in the *cpcB* gene and one in the *cpcA* gene. When the *cpcA* and *cpcB* genes and the IGS between them were analysed separately, we observed that the most polymorphic loci was the *cpcB* gene.

## 6.4.5 FINGERPRINTING METHODS

The ERIC-PCR has been used for fingerprinting and diversity studies of different cyanobacterial species (Rasmussen & Svenning, 1998) and other bacterial genera (Versalovic *et al.* 1991). The use of ERIC-PCR to study the diversity of 18 *Arthrospira* strains shows that

strains belonging to the 4 ITS subclusters may have different ERIC patterns (Fig. 10). Indeed, the ERIC patterns are similar inside the ITS clusters I.A and I.B, but quite heterogenous within clusters II.A and II.B. It is interesting to note that for the 2 strains belonging to cluster II.A/B, one (PCC 7345) has a pattern similar to the one of cluster II.A and the second (DO881) is similar to Cluster II.B. Thus, ERIC-PCR might allow to differentiate *Arthrospira* strains with higher resolution than ITS sequences.

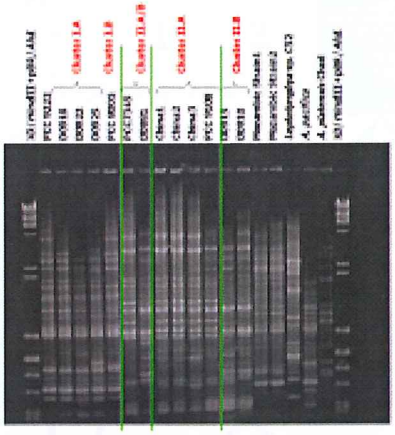


**Fig. 10. Comparison of the ERIC patterns obtained for diferent *Arthrospira* strains belonging to 4 ITS subclusters**

Another fingerprinting method, HIP-PCR, has been successfully used to distinguish cyanobacterial species and strains (Smith *et al.* 1998). Bhaya et al. (2000), described several modified primers based on HIP sequences, from which we have used primer W2HIPACC for differentiation of *Arthrospira* strains. Our preliminary results suggest that this method could be useful for this purpose and has a similar discriminatory power as ERIC-PCR. However, its optimisation is still underway.

Table 1. List of strains, origin, and morphology.

| Strain name | STRAIN | Origin | Morphology | Durham number | Source | Clones in other collections |
|---|---|---|---|---|---|---|
| *Arthrospira* sp. *('platensis')* | LAPORTE 1963/M132-2b | Natron lake, Chad | S | D0906/S | CCALA | |
| *Arthrospira platensis* | SAG 86.79 | Natron lake, Chad | S | D0882 | SAG | Compère 86/79 |
| *Arthrospira* sp. | C1 | Lake Bodou, Kanem, Chad | H (S) | D0918 | A. Sanange-lantoni | |
| *Arthrospira* 'Titicaca' | | Lake Titicaca, Peru | H | D0922 | R. Fox | |
| Commercial product *Arthrospira pacifica* | | | | | B. Meyer | |
| *Spirulina* Tchad | | | | | M. Brouers | |
| *Arthrospira* sp. | SP-9 | Lake Chitu, Ethiopia | | D0892 | DIC | |
| *Arthrospira* sp. | SP-17 | Unknown | H | D0900 | SAC | |
| *Arthrospira* sp. | Strain EF-18A | Unknown | H | D0925 | Earthrise Farms | |
| *Arthrospira* sp. | PCC 9223 | Lake Santa Olalla, Donana National Park, Spain | H | | PCC | |
| *Arthrospira fusiformis* | HEGEWALD 1976/83 | Lake Nakuru, Kenya | S | D0910 | CCALA | |
| *Arthrospira platensis* | PCC 7345 | Saline marsh Del Mar Slough, San Diego Co, CA, USA | H | D0911 | PCC | UTEX 1926; UTEX 1928 ; ATCC 29408 |
| *Arthrospira platensis* | SAG 257.80 | Laguna Huacachina, Ica, Peru | S | D0881 | SAG | Hegewald 1977/229 |
| *Arthrospira* sp | PCC 9108 | Commercial culture facility, Cheng-hai, Yunnan, China | H | D0916 | PCC | |
| *Arthrospira* sp. | PCC 8006 | India, Kenya, Mexico or Peru | H | D0915 | PCC | Records lost at PCC |

H= helical filaments, S: straight filaments, H(S) straight filaments appearing among the helical filaments
ATCC = American Type Culture Collection, Rockville, Maryland, USA
CCALA = Culture Collection of Autotrophic Organisms, Trebon, Czech Republic
Durham = Culture Collection of Durham University, Durham, UK
PCC = Pasteur Culture Collection of Cyanobacterial Strains, Paris, France
SAC = SIAM ALGAE CO., LTD, Samutprakarn,Thailand
SAG = Sammlung von Algenkulturen der Universität Göttingen, Germany
UTEX = Culture Collection of Algae at the University of Texas at Austin, Austin, Texas,

## 6.5. Conclusions

The new molecular markers and fingerprints have confirmed the existence of the 4 subclusters found on the basis of ITS sequences. The polymorphism of *recA* and *cpcBA* gene sequences, and the ERIC fingerprints allowed us to differentiate *Arthrospira* strains belonging to ITS clusters I and II, and between strains from ITS subclusters II.A and II.B, but not between strains from subclusters I.A and I.B. Strains from subclusters I.A and I.B have only 1 base difference in their *rpoC1* and *cpcB-A* genes sequences.

On the basis of the *gyrA* gene sequences, it was possible to differentiate only strains belonging to ITS clusters I and II. In case of *rpoC1* sequences, the difference between clusters I and II.B was clear (ca 30 bp), while it was very small between clusters I and II.A (2 bp - I.A or 3 bp - I.B). Strains PCC7345 and D0881 (II.A/B) have only 1 base difference between each other and are almost identical with strains from cluster II.B (1 bp difference).

Almost all the methods used (excluding *rpoC1* gene sequencing) show that the strain PCC7345 (II.A/B) was identical to strains from cluster II.A., while the strain D0881 (II.A/B) was identical to strains from ITS cluster II.B. Thus, the sequencing of *recA, gyrA, cpcBA* genes and fingerprinting with ERIC allow to differentiate *Arthrospira* strains with a higher resolution than ITS in the case of strains from ITS cluster II.A/B. Only a polyphasic approach gives the best possibility to differentiate these closely related strains belonging probably to the same species.

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# 7. IDENTIFICATION OF GENES RELATED WITH STRESS RESPONSE USING DEGENERATED PCR, BASED ON PROTEIC AND GENOMIC ALLIGNMENT

## *7.1. Introduction*

The possible stress resulting from both space conditions and effluents from the various compartments will be evaluated on the MELiSSA strains. Indeed, these conditions can have an effect on the physiology of the strains and alter the functioning of the whole system. With this aim, the genes known to play an important role in the response to stressful conditions need to be identified.

The expression of genes in bacteria is regulated by environmental conditions. Hence variations in environmental stimuli will result in differential expression of the genes. In order to detect differential gene expression under stress conditions (using enzymatic essays, reporter gene systems, direct RNA quantification, ... ) it is necessary to identify the stress related genes first.

First, the protein sequence of homologous genes present in the databases and identified in various microorganisms, phylogenetically closely and not-so closely related with the MELiSSA strains *R. rubrum* ATCC25903 and *Arthrospira* sp. PCC8005, will be aligned to identify conserved domains in all these proteins. Then, the nucleotide sequence of the corresponding genes will be aligned to allow the design of degenerate oligonucleotides on the basis of the conserved regions in their coding parts. These oligonucleotides will be used to amplify the genes of interest in *Arthrospira* sp. and in *Rhodospirillum rubrum* by PCR. After confirmation of the correct sequence of the amplicons obtained, these PCR amplified DNA fragments will then be used to build biosensors, gene probes or design optimal primers for RNA quantification.

## *7.2. Growing cultures of Arthrospira sp. PCC8005*

Growth of an *Arthrospira* sp. suspension was followed for two different volumes in Zarouk medium (Zarrouk, 1966; TN70.1) at roomtemperature (Table 1). The growth rate was calculated with the following equation: $\mu = dx/dt$, where $\mu$ = specific growth rate ($day^{-1}$), x = initial biomass concentration (OD), and t = time (days).

To calculate the generation time the equation $tg = \ln 2/\mu$ was used, with tg = generation time (days). The mean generation time resides between 2 to 3 days (Table 1). In order to obtain an OD 1 it was therefore necessary to grow the culture for at least 20 days.

| Volume (ml) | Specific growth rate μ ($day^{-1}$) | Generation time tg (days) |
|---|---|---|
| 50 | 0.219 | 3.16 |
| 250 | 0.299 | 2.32 |

**Table 1. Specific growth rate and generation time of *Arthrospira* sp. PCC8005 cultures grown in different volumes.**

## 7.3. Growing cultures of Rhodospirillum rubrum ATCC25903

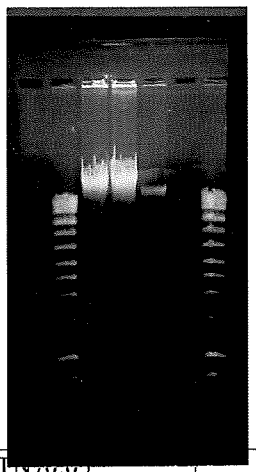
Growth of *R. rubrum* was tested in different media at 30°C in the light (except for 869 and LB) in 5-12 ml volumes. Growth rate and generation time were calculated as above. The tested media were MELiSSA (Segers and Verstraete, 1983), LB (Luria and Bertani, ), 869 (ref) and Sistrom (Sistrom, 1960). Growth rates were comparable with growth rates of *Arthrospira* sp. PCC8005, hence it was likewise necessary to take into account the long incubation times needed to obtain a suspension of certain desired density.

| Medium | Specific growth rate μ ($day^{-1}$) | Generation time tg (days) |
|---|---|---|
| MELiSSA | 0.24 | 2.88 |
| LB | 0.33 | 2.11 |
| 869 | 0.28 | 2.43 |
| Sistrom | 0.25 | 2.80 |

**Table 2. Specific growth rate and generation time of *R. rubrum* ATCC25903 cultures grown in different media.**

## 7.4. Extraction of genomic DNA of Arthrospira sp. PCC8005

Because a 500ml culture of *Arthrospira* sp. PCC8005 needed to grow for 2 months in order to obtain an OD of 1, extraction essays were tested on smaller volumes of 2 ml for DNA extraction with the standard Wizard$^{R}$Genomic DNA kit (Promega), using two lysis solutions (Fig. 1).



**Lane 1** : Smart Ladder 5 μl
**Lane 2** : positive control; *Ralstonia metallidurans* genomic DNA extracted with the Wizard®Genomic DNA kit; 20μl + 4μl blue-orange dye
**Lane 3** : positive control; *Ralstonia metallidurans* genomic DNA extracted with the Wizard®Genomic DNA kit; 20μl + 4μl blue-orange dye
**Lane 4** : *Arthrospira* sp. genomic DNA extracted with the Wizard®Genomic DNA kit 20μl + 4μl blue-orange dye
**Lane 5** : *Arthrospira* sp. genomic DNA extracted with the Wizard®Genomic DNA kit 20μl + 4μl blue-orange dye; replacement of the standard lysis solution by solA (25% sucrose, 10 mM EDTA, 50 mM Tris HCl, pH7.5)
**Lane 6** : Smart Ladder 5 μl

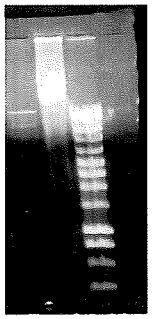
**Fig. 1. Total genomic DNA extraction of *R. metallidurans* CH34 and *Arthrospira* sp. PCC8005 with the Wizard®Genomic DNA kit.**

A Japanese group used the kit under the same conditions for genomic DNA extraction of the related cyanobacterium *Spirulina platensis* M-135, for the construction of a genomic library (Kawata et al. 1998).
Our results show that the kit was not usefull for DNA extraction of *Arthrospira* sp. PCC8005 (Fig. 1). On the other hand the DNA extracted from a 500 ml culture according to Vonshak (1997), revealed high quantities of almost pure DNA after electrophoresis (Fig. 2).

## *7.5. Extraction of total genomic DNA by Vonshak (1997)*

1. Grow a culture of 500 ml until OD 1 at 600 nm.
2. Centrifuge the culture 2-3 times at 12000 rpm during 10 min at 4°C.
3. Wash the pellet one time with double distilled sterile water by resuspension and centrifugation at 12000rpm for 10 min.
4. Resuspend 2ml of the lysis buffer (25% sucrose, 10 mM EDTA, 50 mM TrisHCL, pH 7.5).
5. Incubate the suspension at -70°C for 30 min.
6. Incubate the sample at 37°C for 20 min.
7. Add lysozyme to a final concentration of 1mg/ml and incubate at 37°C for 30 min.
8. Add SDS (final concentration 10 mg/ml) and proteinase K (final concentration 0.1 mg/ml). Mix by inversion.
9. Incubate overnight in a water bath at 55°C.
Add the same volume of a phenol-chloroform-isoamylalcohol (25:24:1). Mix gently by inversion.
10. Centrifugate for 10 min at 12000 rpm. Save the supernatant.
11. Treat the sample with RNAse (final RNAse concentration 50 µg/ml) during 1 hour at 37°C.
12. Extract the sample again with phenol-chloroform-isoamylalcohol (25:24:1). Take the aquaous phase.
13. Add a volume equivalent of chloroforme-isoamylalcohol (24:1). Mix gently for 10-15 min.
14. Centrifuge for 10 min at 12000 rpm. Take the supernatant.
15. Add sodium acetate to a concentration of 0.3 M to precipitate the chromosome.
16. Mix until DNA becomes visible.
17. Precipitate the DNA with ethanol by adding two volumes of cold ethanol.
18. Make a little hook at the end of a sterile pasteur pipet. Catch the DNA by turning the hook inside the sample.
19. Incubate the hook, containing the DNA, afterwards inside eppendorfs containing 70% and 100% for two seconds in order to wash the DNA.
20. Air dry the DNA for 30 min by opening the eppendorf to allow evaporation of the remaining ethanol.
21. Resuspend the hook containing the DNA in 500µl of TE buffer (10 mM TrisHCl pH8, 1mM EDTA).

22. Let the DNA dissolve at room temperature first. If necessary incubate the tube for 15 min at 65°C to enhance dissolving. If there is still to much DNA attached to the hook, buffer can be added up to 2ml total volume and a little incubation at 65°C for 15 min can be repeated.
23. Estimate DNA concentration.
24. Store DNA at -20°C in aliquots.



**Lane 1** : *Arthrospira* sp. genomic DNA extracted with the method of Vonshak; 20µl + 4µl blue-orange dye
**Lane 2** : Smart Ladder 5 µl

**Fig. 2. Total genomic DNA extraction of *Arthrospira* sp. PCC8005 using the method of Vonshak.**

## *7.6. Selection of the target genes*

The selected genes should play a fundamental role in the general mecanisms of resistance to divers stresses. The nature of the stress is dependant on the conditions that will most likely occur in the MELiSSA loop (temperature stress, nitrogen deprivation, ...) as well as environmental stresses related to space (oxydative stress, UV irradiation, cosmic irradiation, ...). According to the literature that most of the target stress related genes are relatively high conserved among closely as well as distantly related organisms of *Arthrospira* sp. and *Rhodospirillum rubrum*. It is therfore reasonable to assume that the proteic and/or nucleic sequences will present enough homologie among bacterial species to design degenerate primers in an attempt to amplify the target genes from *Arthrospira* sp. and *Rhodospirillum rubrum*.

For the design of degenerate primers on *Arthrospira* sp. it was possible to use the sequence of phylogenetically closely related strain *Synechocystis* sp. PCC6803. Likewise, other cyanobacterial sequences have been used during design of the degenerate primers.

For *Rhodospirillum rubrum*, two purple non-sulfur, relatively close related species, bacteria (*Rhodobacter sphaeroides* and *Rhodobacter capsulatus*) have been considered for the design of the degenerate primers.

| **Type of stress** | **gene** | **Protein** |
|---|---|---|
| **Thermic stress** | *rpoH* | Sigma 32 |
| | *sigB* | SigmaB or Sigma 37 |
| | *dnaK* | dnaK |
| | *dnaJ* | DnaJ |
| | *groEL* | GroEL |
| | *groES* | GroES |

| | | |
|---|---|---|
| | *clpB* | ClpB |
| | *clpX* | ClpX |

| | | |
|---|---|---|
| **Oxydative stress** | *oxyR* | OxyR |
| | *soxR* | SoxR |
| | *soxS* | SoxS |
| | *mrsA* | Methionine sulfoxide reductase |
| | *sodA* | Magnesium superoxyde dismutase |
| | *sodB* | Iron superoxyde dismutase |
| | *katE* | Catalase HPII |
| | *katG* | Catalase HPI |
| | *katA* | Catalase |
| | *perA* | Catalase-Peroxydase |
| **General stress (stationary phase)** | *rpoS* | SigmaS |
| **UV stress** | *recA* | RecA |
| | *lexA* | LexA |
| | *uvrA* | UvrA |
| | *mutS* | MutS |
| **Nitrogen deprivation** | *glnA* | Glutamine synthetase |

**Table 1: List of target genes and proteins selected to be investigated in *Arthrospira* sp. PCC8005 and *Rhodospirillum rubrum* ATCC25903.**

Listed above, are the genes (and proteins) selected on the basis of their interspecies conservation for each selected type of stress (Table 1). At this stage no attempt was made in creating an exhaustive list of genes implicated in stress response. The list is teherfore limited to 24 genes relatively well known in scientific literature.

Before testing the possibility of designing degenerated oligonucleotides specific for a certain gene, the conservation of the proteic sequence is first verified. De homology of the sequences (in FASTA format on the website http://www.expasy.org/) of each protein was tested on *Eschericia coli* or *Ralstonia metallidurans* (reference gram-negative bacteria), *Rhodobacter sphaeroides* and/or Rhodobacter capsulatus (reference bacteria for Rhodospirillum rubrum), whereafter the homologous proteeic sequence fragments were compared at the genomic level (translated on the site http://www.doe.jgi.org/) (Table 2).

Proteic sequences were also compared on the site http://bioweb.pasteur.fr/seqanal/blast/ between the gram-negative reference strains (Escherichia coli or Rhodobacter sphaeroides) and a reference gram-positive strain Bacillus subtillis, in order to confirm the choice of proteins induced during stress response (Table 2).

| | Protein identity % | | | | | |
|---|---|---|---|---|---|---|
| | **DnaK** | **GroEL** | **ClpB** | **ClpX** | **RecA** | **Catalase** |
| ***E. coli* - *R. sphaeroides*** | 68 | 62 | 54 | 71 | 66 | 64 |
| ***E. coli* – *R. capsulatus*** | 83 | 81 | | | | |
| ***E. coli* – *B. subtilis*** | 62 | 71 | | 67 | | 63 |
| ***R. sphaeroides* – *B. subtilis*** | 54 | 59 | | 60 | | |
| ***E. coli* – *R. metallidurans*** | | | 63 | | 68 | |

**Table 2. Percentage of protein identity among the reference strains.**

An alignement of protein sequences of all proteins, retained from *E. coli* was accomplished on the NCBI site with all proteic sequences present at the database, thereby enabling the possibility of detecting higly conserved regions in the investigated protein sequences.

## *7.7. Results of the proteic and nucleic sequence alignment*

Multiple alligments between the proteic sequences of the same protein synthesized by different phylogenetically closely related as well as distantly related micro-organisms were accomplished using website http://npsa-pbil.ibcp.fr./.

A few examples of the allignement results are presented below. On the Fig. 3-7 you can find the allignements for the proteins DnaK, Catalase (*katE*), UvrA and MsrA.
The regions containing 6 successive conserved amino acids are encased.

**Fig. 3. Extract of the allignment of the protein sequence of DnaK in phylogenetically distantly related organisms (*Agrobacterium tumefaciens*, *Caulobacter crescentus*, *Rhodobacter capsulatus*, *Alcaligenes eutrophus*, *Buchnera aphidicola*, *Escherichia coli*, *Chlamydia trachomatis*, *Bacillus subtilis*, *Anabaena* sp., *Aquifex pyrophilus*) (http://npsa-pbil.ibcp.fr) (fragments 10-240 and 330-400), with a mean total lenght of the protein sequence: 700 amino acids.**

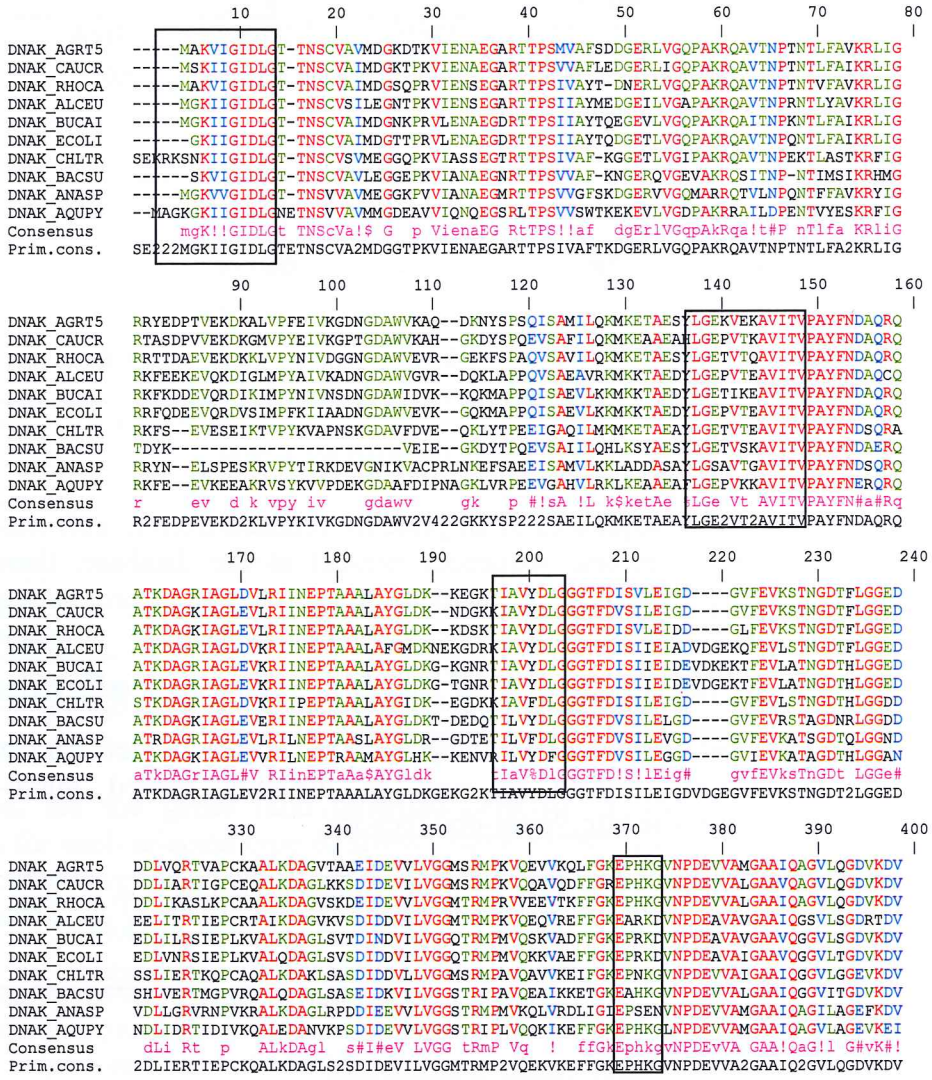


**Fig. 4. Extract of the allignment of the protein sequence of DnaK in organisms phylogenetically closely related to *Arthrospira* sp. PCC8005 (*Synechocystis* sp. 6803 who possesses 3 isoforms of protein DnaK and *Synechococcus*) (http://npsa-pbil.ibcp.fr/), with a mean total length of 800 amino acids.**

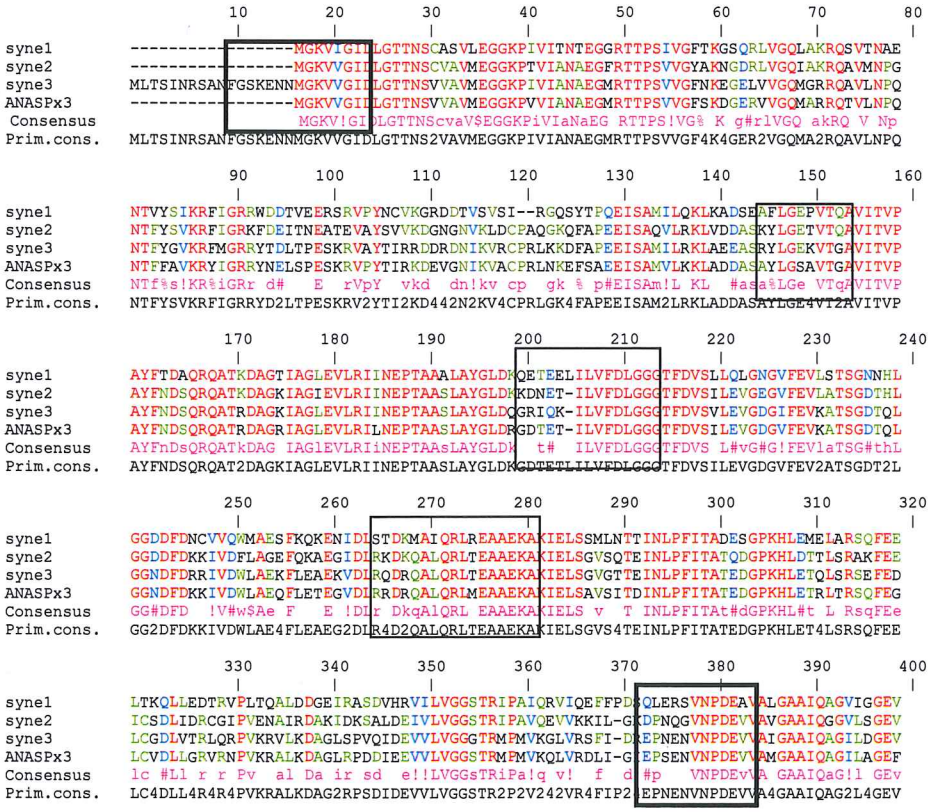


**Fig. 5. Extract of the allignment of the protein sequence of catalase (encoded by *katE*) in phylogenetically distantly related organisms (*Rhizobium melitoti*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Mycobacterium avium*, *Bacillus subtilis*) (http://npsa-pbil.ibcp.fr/), with a mean total length of 770 amino acids.**

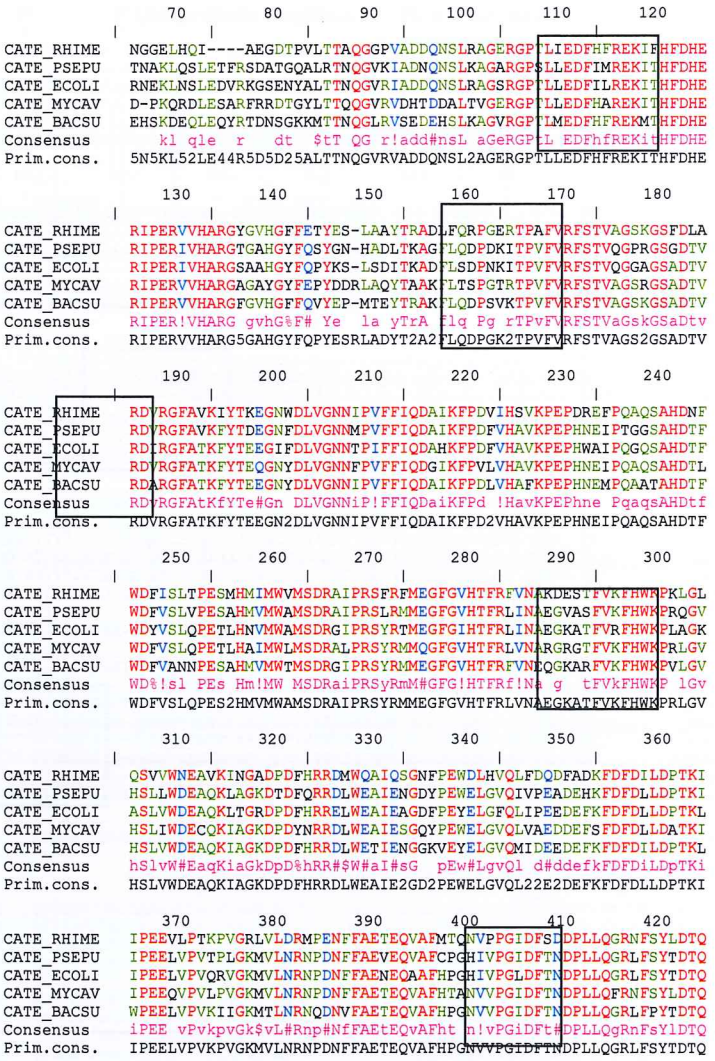


**Fig. 6. Extract of the alignement of the protein sequence of the protein UvrA in organisms, phylogenetically closely related to *Arthrospira* sp. (*Synechocystis* sp. PCC6803 and *Anabaena*) (http://npsa-pbil.ibcp.fr/), with a mean total lenght of 990 amino acids.**

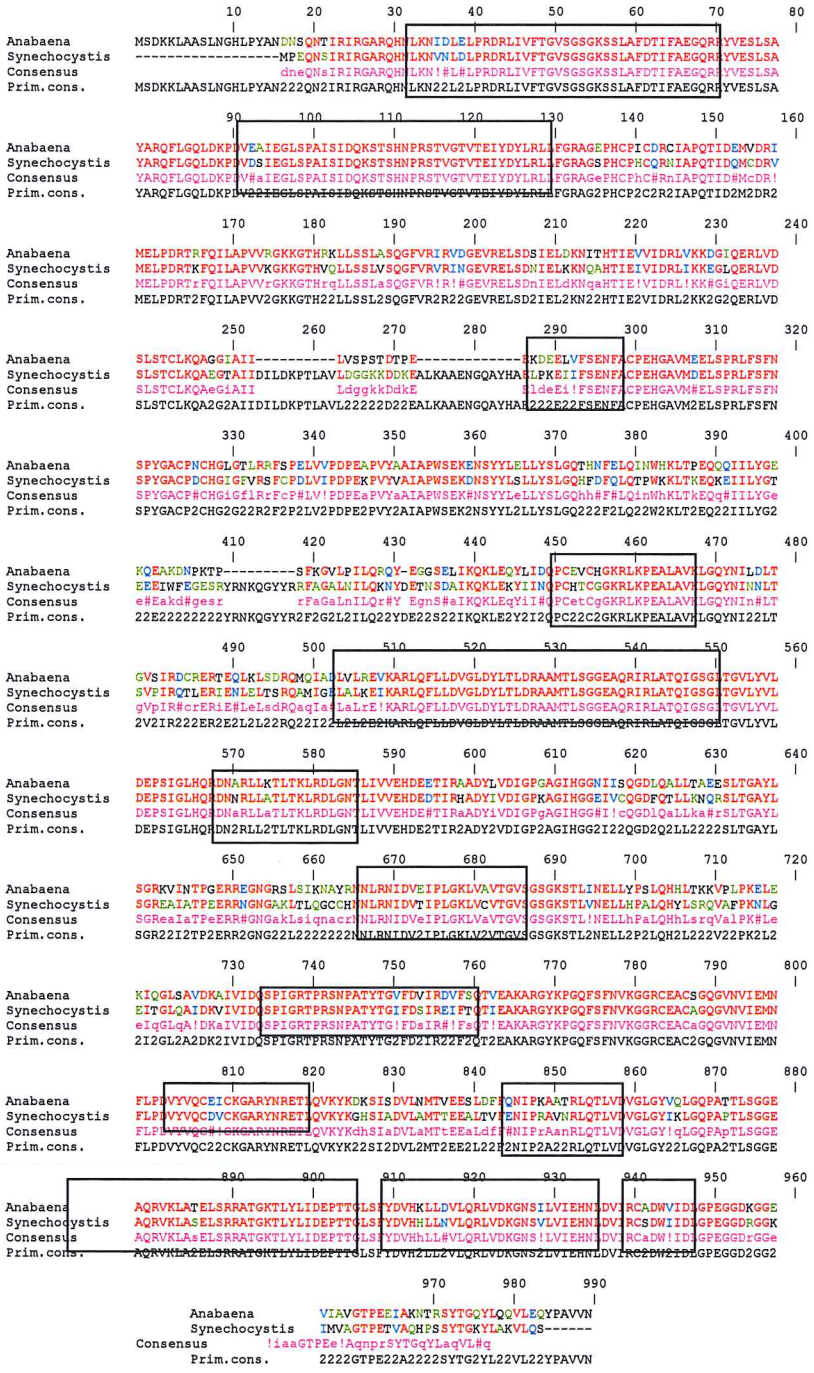


**Fig. 7. Extract of the alignment of the protein sequence of the protein MsrA in phylogenetically distantly related organisms (*Anabaena* sp., *Caulobacter crescentus*, *Agrobacterium tumefaciens*, *Escherichia coli*, *Clostridium perfringens*, *Methanobacterium thermocellum*, *Deinococcus radiodurans*) (http://npsa-pbil.ibcp.fr/), with a mean total length of 180 amino acids.**

```
                    10        20        30        40        50        60
           |        |         |         |         |         |
MSA1_ANASP  ------------------------------------------------------GHAEV
MSA1_CAUCR  ----------------------VFWKVPGVYVTAAGYAAGITPNPTYEEVCTGRTGHTEV
Agrobacterium -------EIIYLGMGCFWGAERLFWKTPGVWVTAVGYAGGFTRNPTYHETTTGQTGHAEV
MSRA_ECOLI  ------------------------QLPGVYSTAAGYTGGYTPNPTYREVCSGDTGHAEA
MSRA_CLOPE  ------MKKIILAGGCFWGVEEFLSRINGVVSTEVGYANGRTENPTYEDICTKNTYFAEV
MSRA_METTH  MMCLSRYEKATFGAGCFWGVEDAFRKVDGVVSTRVGYMGGHLENPTYEDVCTGLTGHAEV
MSRA_DEIRA  ----------------------------------GYIGGTVPNPDYRSVCGGQTGHAEA
Consensus                                      gv  t  gy gg   npty  vc g tghAEv
Prim.cons.  MMCLSR2EKI3LG3GCFWGVE34FWKVPGV2STAVGYAGG6TPNPTYEEVCTGQTGHAEV

                    70        80        90       100       110       120
           |        |         |         |         |         |
MSA1_ANASP  VQIEYDPQLVSYEDLLAVFWDIHDPTTLNRQGPDKGEQYRSVIFFHNEQQAEAAQQSKAK
MSA1_CAUCR  VLVVFDPKVVTYEALLKTFWENHDPTQGMRQGNDIGTQYRSGLYVTSDAQAAAAAESKAA
Agrobacterium VKVVYDPAVISLSGLLKIFFEEHDPTQGMRQGNDVGTTYRSAIYATTEGQLQQAQKARDA
MSRA_ECOLI  VRIVYDPSVISYEQLLQVFWENHDPAQGMRQGNDHGTQYRSAIYPLTPEQDAAARASLER
MSRA_CLOPE  CLVNYDENIISLKELLAKFWTIIDPTSLNKQGNDVGSQYRTGIYYVDSSDLEDILNSKEE
MSRA_METTH  VEVTFDPDVVGYSDLLDVFWSIHDPTTLNRQGPDVGEQYRSVIFYHSDEQRRAAIESRRR
MSRA_DEIRA  VRVTFDPNQISYRDLLGLFFATHDPTSLNRQGADVGTQYRSALFPLTQEQEQTAREMIEQ
 Consensus    v ! %Dp  !sy dLL vFw ihDPt lnrQG DvG QYRs i%     #  aa  s
Prim.cons.  V2VVYDPNVISYEDLL2VFWEIHDPTQLNRQGNDVGTQYRSAIY23T2EQ23AA2ESKE2

                   130       140       150       160       170       180
           |        |         |         |         |         |
MSA1_ANASP  VQVSGRFEL---DIVTEIKHKSEYYLATEEHQQYFEKQAKR-------------------
MSA1_CAUCR  YQQALSARGLG-TITTEIAPAGPFYFAEDYHQQYLAKNP-NGYCGIGGTGVVCPIGLGVE
Agrobacterium FQQALDEAGHGHAITTEIGPLETFYYAEDYHQQYLAKNP-GGYCGLRGTGVSCNIG----
MSRA_ECOLI  FQAAMLAADDDRHITTEIANATPFYYAEDDHQQYLHKNP-YGYCGIGGIGVCLPPEA---
MSRA_CLOPE  LQKS--YSK---KIVTEVKPLENYYKAEEYHQKYLKKNP-NGYCHIKLD-----------
MSRA_METTH  LEESGRFRD---RIVTAIEPAGTFYEAEEYHQQYLEKNP-RRRCYLMRLLSTR-------
MSRA_DEIRA  LGTEDVFGR---PIVTSIEPASTFYVAEDYHQNYYKNNPGQGYCMAVISPKVAKLRQYYG
       Consensus        q s  f      IvTe! pa  %Y Ae#yHQqYl k#p  gyc
Prim.cons.  LQQ22RF223G27IVTEI3PA3TFYYAEDYHQQYL3KNP22GYCGIGGTGVVCPIG3222

                    MSA1_ANASP      ----
                    MSA1_CAUCR      G---
                    Agrobacterium   ----
                    MSRA_ECOLI      ----
                    MSRA_CLOPE      ----
                    MSRA_METTH      ----
                    MSRA_DEIRA      DKLR
                         Consensus
                    Prim.cons.      2KLR
```

In the alignment of the protein sequences of DnaK of the organisms closely related to *Arthrospira* sp. it was possible to detect much longer conserved regions compared with the allignement of DnaK between more distantly related organisms (Fig. 4.). In the alignement of the protein sequence of UvrA of organisms closely related to *Arthrospira* sp., it is observed that the sequences are very strongly conserved (Fig. 5). In this case, it was therefore possible to choose the most optimal position based on the conservation of the gene at the genetic level and still keep the level of degeneracy of the primers very low.

**Fig. 8. (see next page) Extract of the nucleic acid sequence alignment of *dnaK* (*Agrobacterium tumefaciens*, *Caulobacter crescentus*, *Rhodobacter capsulatus*, *Alcaligenes eutrophus*, *Buchnera aphidicola*, *Escherichia coli*, *chlamydia trachomatis*, *Bacillus subtilis*, *Anabaena* sp., *Aquifex pyrophilus*) (on website http://npsa-pbil.ibcp.fr/); mean lenght of 1870 bases.**

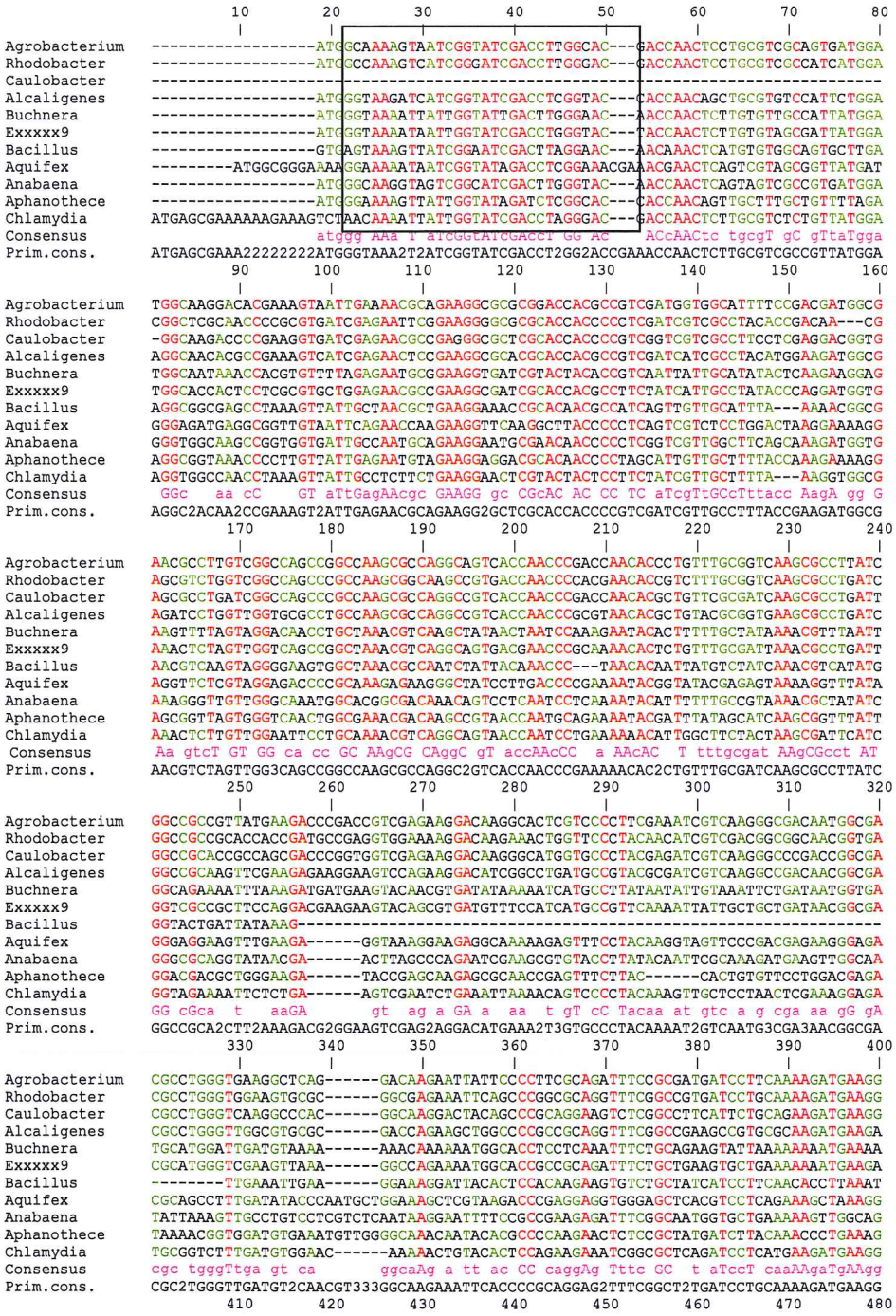
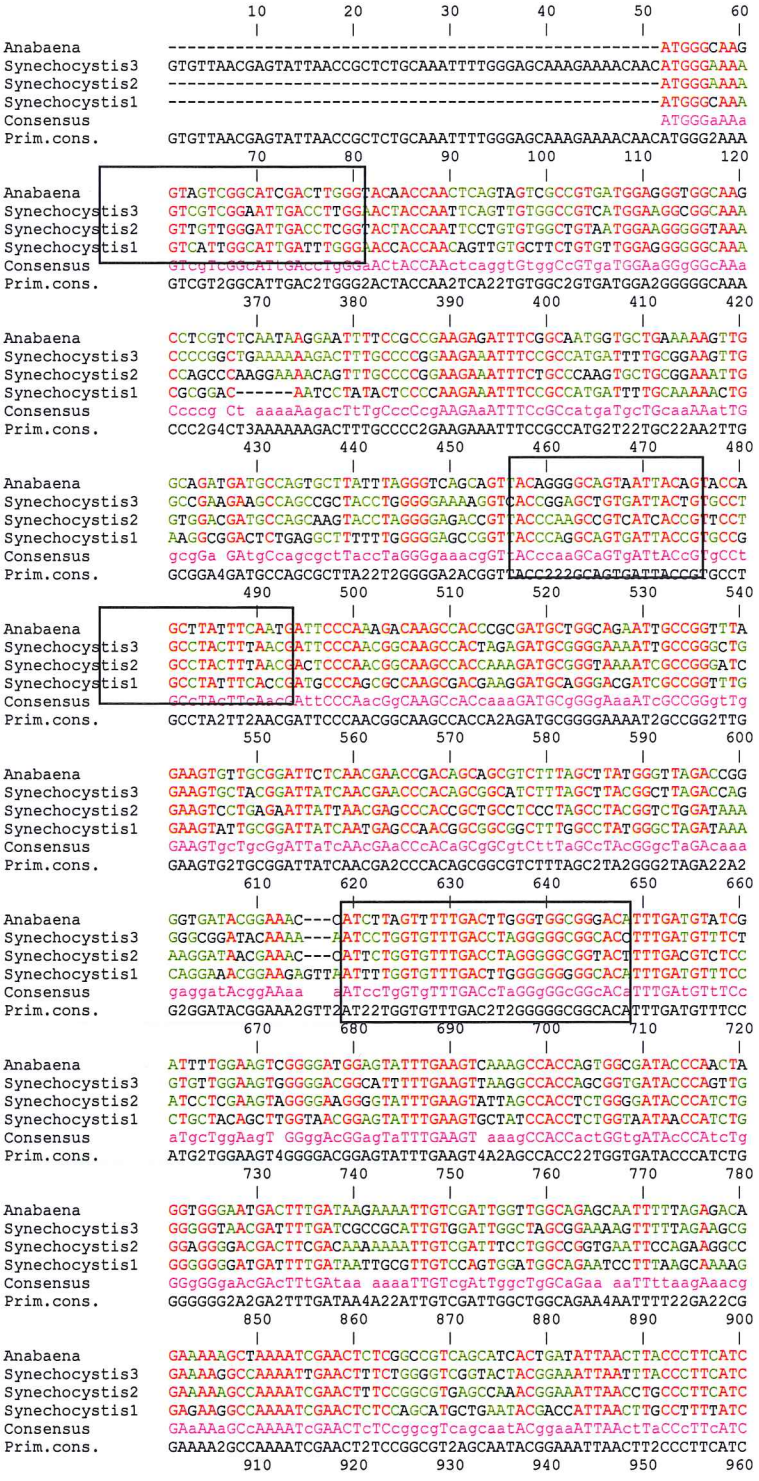


**Fig. 9. Extract of the nucleic acid sequence alignment of *dnaK* in organisms closely related with *Arthrospira* sp. (*Anabaena* and *Synechocystis* sp. PCC6803) (on website http://npsa-pbil.ibcp.fr/); mean length of 2570 bases.**




For the alignment of MsrA between distantly related organisms no conserved region of sufficient length was found (Fig. 7). It was likewise impossible to obtain conserved regions of sufficient length when organisms that are closely related to *Arthrospira* sp. were used (i.e. *Synechocustis* sp. PCC6803 and *Anabaena*) (results not shown). In order to be able to design appropriate degenerate oligonucleotides, a conserved region of at least 7 amino acids should be found residing at a convenient distance between each other.

Of the 24 stress related proteins, selected on the basis of the literature, 7 proteins were discarded because they presented a homology that was too low to create reasonable degenerate primers. The proteins that were discarded were: Sigma432, OxyR, SoxR, SoxS, MrsA, catalase-peroxydase (*perA*), MutS (see MrsA, Fig. 7).

The other proteins (DnaK, DnaJ, GroEL, GroES, ClpB, ClpX, SigmaB, SOD-Fe, SOD-Mn, Ctalase-Peroxidase HPI encoded by katG, Catalase-Peroxydase HPII encoded by *katE*, Catalase encoded by *katA*, RpoS, UvrA, RecA, LexA, GlnA) presented enough conserved regions when the protein sequences were aligned between organisms distantly as well as closely related.

Based on the conserved protein sequences, the nucleotide regions that contained the highest conservation were selected. In these cases it was possible to select for a region of an appropriate length, flanked with conserved regions of 20-25 nucleotides (see the rules for designing degenerate primers TN70.3). The regions on the nucleotide sequence to which the degenerated primers would anneal are encased in the next figures.

**Fig. 10. Extract of the nucleic acid sequence allignement of *katE* of distantly related organisms (*Rhizobium melitoti*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Mycobacterium avium*, *Bacillus subtillis*) (on website http://npsa-pbil.ibcp.fr/); mean length of 2320 bases.**

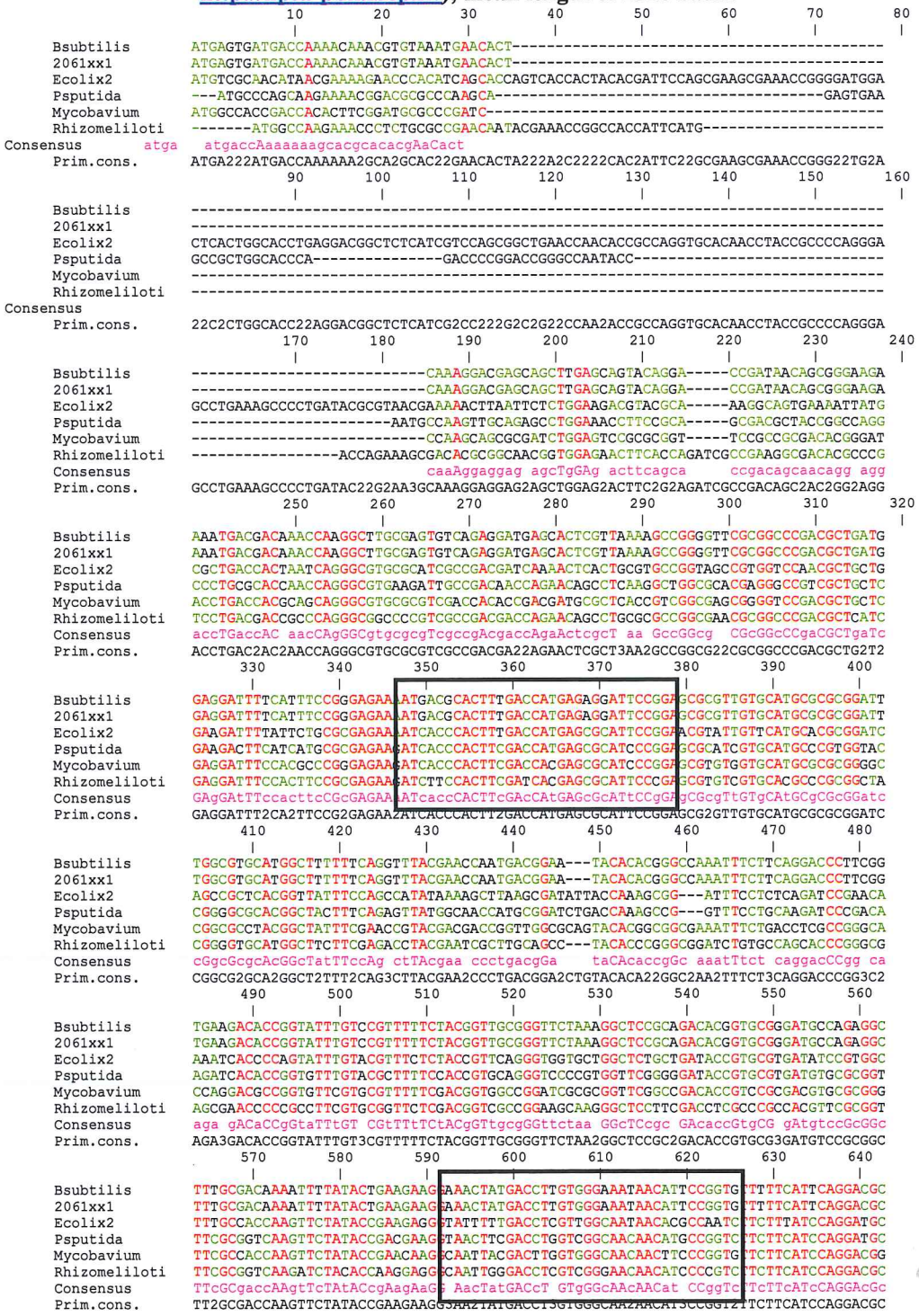
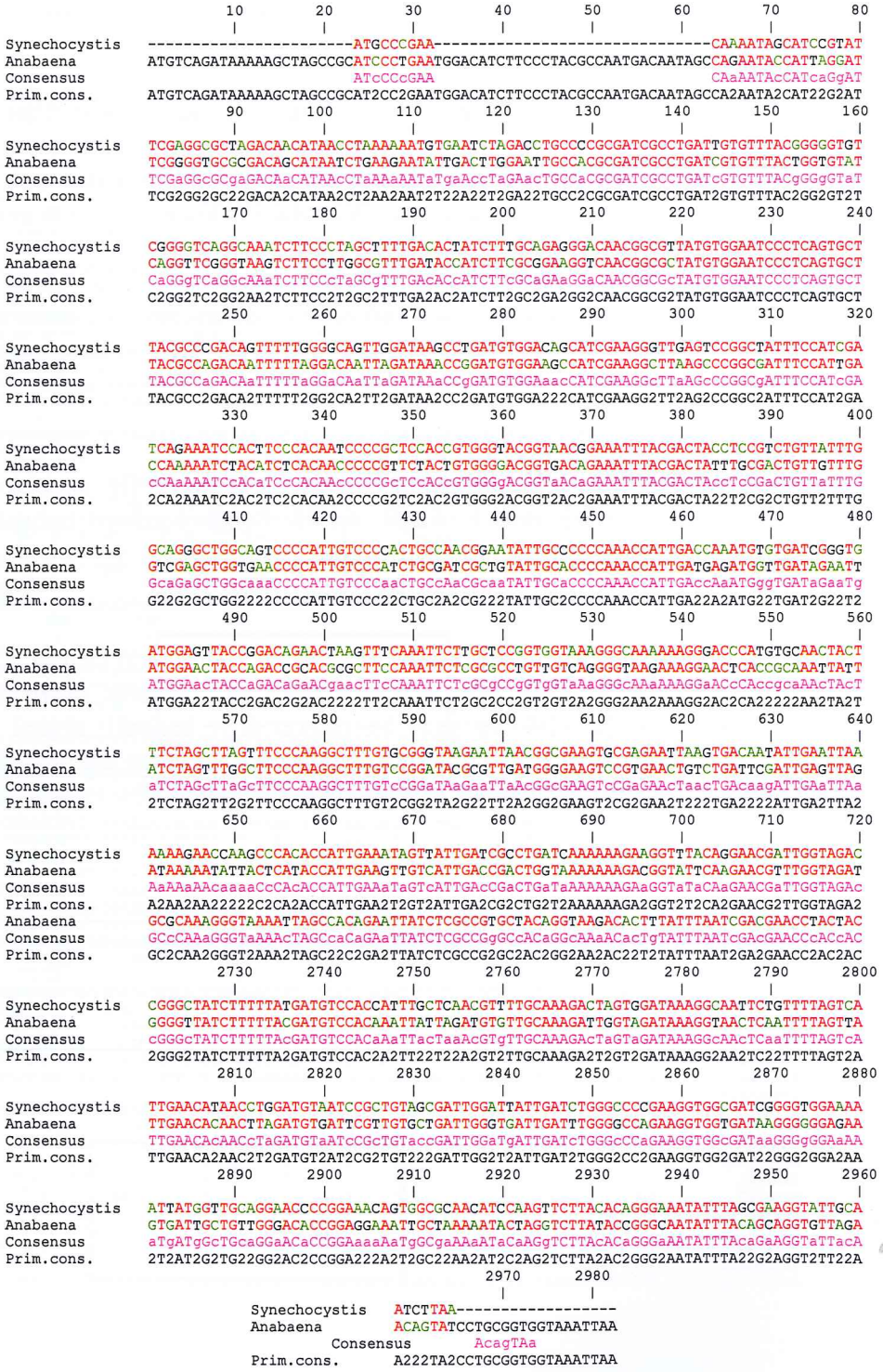
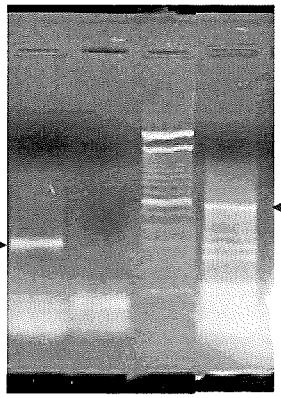


**Fig. 11. Extract of the nucleic acid sequence alignment of uvrA in microrganisms closely related with *Arthrospira* sp. (*Synechocystis* sp. PCC6803 and *Anabaena*) (http://npsa-pbil.ibcp.fr/); mean length of 2980 bases.**



When the alignment of the gene *uvrA* was investigated in organisms closely related to *Arthrospira* sp., it could be observed that any region of 20 to 25 nucleotides could be chosen for designing suitable degenerate primers (Fig. 11).

**Fig. 12. Gel electrophoresis of PCR products obtained with degenerate primers selective for $katE_{94\text{-}95}$ and $dnaJ_{8\text{-}10}$ on *R. rubrum* genomic DNA (PCR annealing conditions: 5 cycles of 37°C; 40 cycles of 57.6°C and 60.2°C respectively using $katE_{94\text{-}95}$ and $dnaJ_{8\text{-}10}$); expected band for $katE_{94\text{-}95}$ at 290bp and $dnaJ_{8\text{-}10}$ at 528bp.**

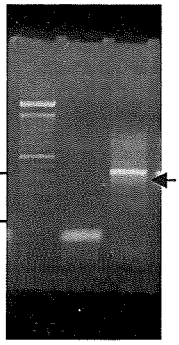


200pb

600pb

**Lane 1** : PCR amplicon obtained with degenerate primer mix selective for *katE* $_{94\text{-}95}$ (expected band at 290 pb)
**Lane 2** : no DNA; negative control
**Lane 3** : 100bp DNA Ladder
**Lane 4** : PCR amplicon obtained with degenerate primer mix selective for *dnaJ* $_{8\text{-}10}$ (expected band at 528 pb)

**Fig. 13. Gel electrophoresis of PCR products obtained with degenerate primers selective for $sodB_{62\text{-}69}$ on *Arthrospira* sp. genomic DNA (PCR annealing conditions: 5 cycles at 37°C; 40 cycles at 58.1°C); expected band for $sodB_{62\text{-}69}$ at 426bp.**

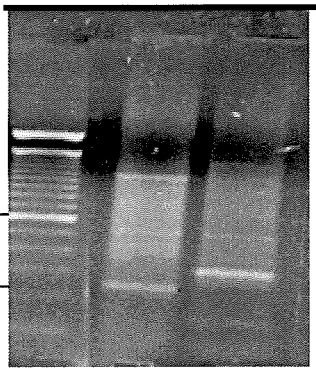


400pb

100pb

**Lane 1** : 100 pb DNA Ladder
**Lane 2** : no DNA, negative control
**Lane 3** : PCR amplicon obtained with degenerate primer mix selective for *sodB* $_{62\text{-}69}$ (expected band at 426 pb)

**Fig. 14. Gel electrophoresis of PCR product obtained with degenerate primers selective for $katA_{92\text{-}93}$ and $uvrA_{105C\text{-}105A}$ on *Arthrospira* sp. genomic DNA. (PCR annealing conditions: 45 cycles at 51.7°C and 51.3°C respectively using $katA_{92\text{-}93}$ and $uvrA_{105C\text{-}105A}$)**




**Lane 1** : 100 bp DNA Ladder
**Lane 2** : PCR amplicon obtained with degenerate primer mix selective for $katA_{92\text{-}93}$ (expected band at 257 pb)
**Lane 3** : PCR amplicon obtained with degenerate primer mix selective for $uvrA_{105C\text{-}105A}$

The last series of alignments enabled the design of a number of oligonucleotide couples for every targeted gene for *Arthrospira* sp. as well as *R. rubrum*. In total 81 oligonucleotide couples were defined (see Appendix 1). Among these, 36 of the most representative primer pairs were selected and tested.

## 7.8. Results of the degenerated PCR

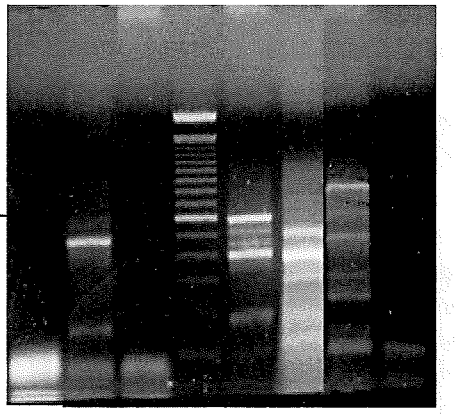
Due to the temperature (37°C) used during the first 5 annealing cycles initial aspecific annealing occurred, resulting in the appearrance of amplification smears, except for $katE_{94\text{-}95}$ (amplification using the degenerated primer pair 94 and 95, Appendix 1) for both *Arthrospira* sp. and *Rhodospirillum rubrum* (Fig. 12). Strongly present single bands were also obtained with $sodB_{62\text{-}69}$ in *Arthrospira* sp. (Fig. 13) and for $uvrA_{101\text{-}103}$ and $dnaJ_{8\text{-}10}$ (Fig. 12) in *Rhodospirillum rubrum*.

To obtain more specificity, annealing PCR conditions were changed into 45 cycles at the mean estimated annealing temperature. In this case a PCR profile of a number of bands is obtained, among which the band of interest is more visible against a background of aspecific PCR amplification bands (Fig. 14, 15). An attempt to increase selection of the bands of interest by nested PCR, did not result in an increase of specific DNA amplification.

On the 36 tested primer pairs, 10 have resulted in selective amplification with the correct sequence length: $dnaK_{3\text{-}6}$, $dnaJ_{12\text{-}14}$, $sodA_{70\text{-}72}$, $sodB_{62\text{-}69}$, $katA_{92\text{-}93}$, $katE_{94\text{-}95}$, $uvrA_{105C\text{-}105A}$ and $uvrA_{101\text{-}103}$ for *Arthrospira* sp.; $katE_{94\text{-}95}$, $clpB_{35\text{-}36}$ and $uvrA_{101\text{-}103}$ voor *R. rubrum* (Fig. 14, 15).

Further PCR optimisation was done order to obtain higher specificity. For the couples of *Arthrospira* sp. $dnaK_{3\text{-}6}$ and *R.rubrum* $clpB_{35\text{-}36}$ a specific band with the correct length was obtained using touch-down PCR and additional PCR under the same conditions (Fig. 16).

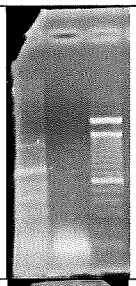
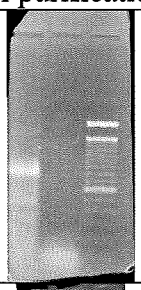
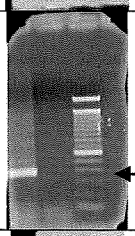
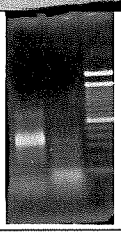
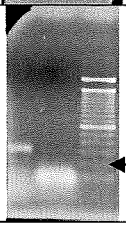
**Fig. 15. Gel electrophoresis of PCR product obtained with degenerate primers selective for *uvrA*$_{101-103}$ for *Arthrospira* sp. and *R. rubrum* genomic DNA; *dnaJ*$_{12-14}$ and *dnaK*$_{3-6}$ for *Arthrospira* sp. genomic DNA; and *clpB*$_{35-36}$ for *R. rubrum* genomic DNA. (PCR annealing conditions: 45 cycles at 62.8°C, 67.8°C, 55.6°C and 67.7°C for *uvrA*$_{101-103}$, *dnaJ*$_{12-14}$, *dnaK*$_{3-6}$ and *clpB*$_{35-36}$).**



0 pb

**Lane 1** : no DNA, negative control
**Lane 2** : PCR amplicon obtained with degenerate primer mix selective for *uvrA* $_{101-103}$ from *Arthrospira.* sp. genomic DNA (expected band at 583 bp)
**Lane 3** : no DNA, negative control
**Lane 4**: 100bp DNA Ladder
**Lane 5** : PCR amplicon obtained with degenerate primer mix selective for *dnaJ* $_{12-14}$ from *Arthrospira.* sp. genomic DNA (expected band at 443 pb)
**Lane 6** : PCR amplicon obtained with degenerate primer mix selective for *clpB* $_{35-36}$ from *Arthrospira.* sp. genomic DNA (expected band at 389 pb)
**Lane 7** : PCR amplicon obtained with degenerate primer mix selective for *dnaK* $_{3-6}$ from *Arthrospira.* sp. genomic DNA (expected band at 794 pb)
**Lane 8** : no DNA, negative control

**Fig. 16. Gel electrophoresis of PCR product obtained with degenerate primers selective for *dnaK*$_{3-6}$, *clpB*$_{35-36}$, and *katE*$_{94-95}$, using touch down PCR and followed by a second PCR on the extracted band obtained from the first PCR round.**

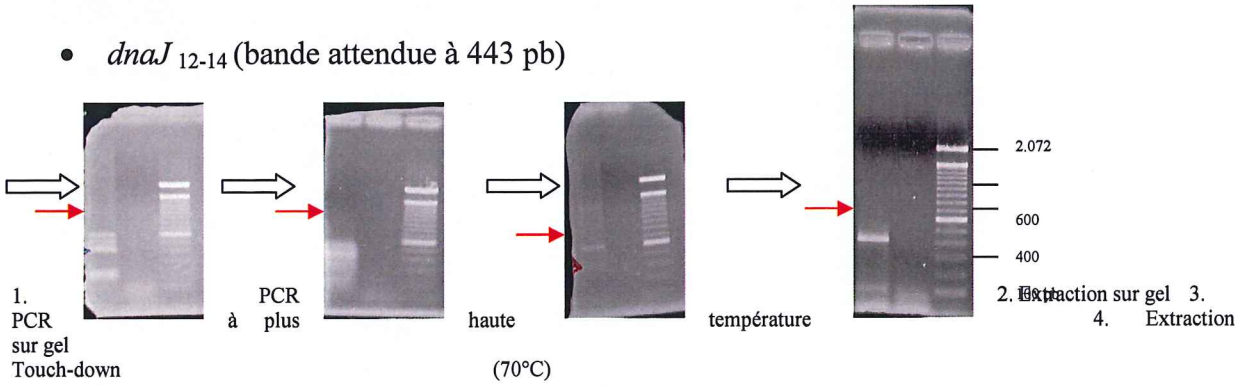
| | After PCR "Touch-Down" | After gel purification |
|---|---|---|
| *dnaK* $_{3-6}$ *(A.platensis)*<br><br>=> band expected at 794 pb |  |  ◄2.072 ◄1.500 ◄600 ◄100pb |
| *clpB* $_{35-36}$ *(R.rubrum)*<br><br>=> band expected at 389 pb |  ◄ 300pb |  |
| *katE* $_{94-95}$ *(R.rubrum)*<br><br>=> band expected at 290 pb |  ◄ 200pb | |

**Ligne 1** : PCR amplicon; **Ligne 2** : negative control (no DNA); **Ligne 3** : 100 bp DNA Ladder

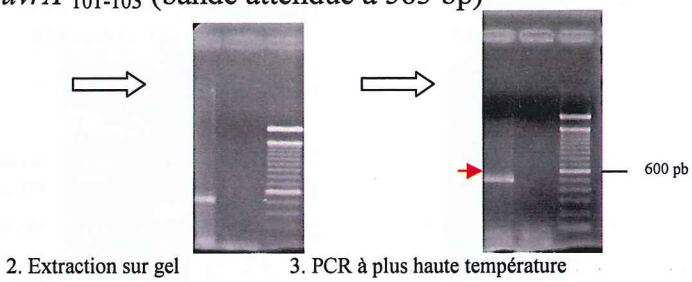
PCR optimisation with touchdown PCR did not improve selective application of *dnaJ*$_{12\text{-}14}$ and *sodA*$_{70\text{-}72}$. An alternative optimisation method was thereafter selected. In this case the highest estimated annealing temperature was used in the PCR program. Using the elevated annealing temperature, a specific band of the expected sequence length was obtained for *dnaJ*$_{12\text{-}14}$ and *uvrA*$_{101\text{-}103}$ in *Arthrospira* sp. (Fig. 16).

**Fig. 17. Optimisation of the degenerative PCR selective for *dnaJ*$_{12\text{-}14}$, *uvrA*$_{101\text{-}103}$ on *Arthrospira* sp. genomic DNA.**

- *dnaJ* $_{12\text{-}14}$ (bande attendue à 443 pb)




- *uvrA* $_{101\text{-}103}$ (bande attendue à 583 bp)




Optimisation of PCR by adjusting ratio's of degenerate primer pairs could improve selective amplification of sodB$_{62\text{-}69}$ on *Arthrospira* sp. genomic DNA (Fig. 18).

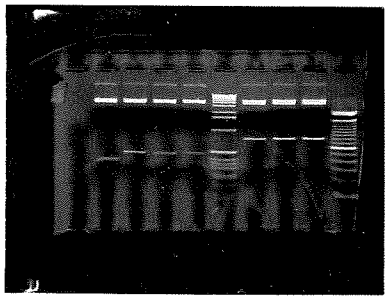
**Fig. 18. Optimisation of the degenerative PCR selective for *sodB*$_{62\text{-}69}$ on *Arthrospira* sp. genomic DNA.**

**Lane 1** : PCR amplicon obtained with degenerate primer mix 62 (with 19% degeneracy) and 69 (with 4.76%) in a 1:1 ratio.
**Lane 2** : : PCR amplicon obtained with degenerate primer mix 62 and 69 in a 10:1 ratio.

Direct sequencing of the DNA bands extracted from the gel after amplification with *sodB*$_{62\text{-}69}$ and *dnaJ*$_{12\text{-}14}$ did not give satisfying results. It seemed that the products contained too much additional contaminating amplicons. It was therefore necessary to clone the PCR products, prior to sequencing. The products of *dnaK*$_{101\text{-}103}$, *clpB*$_{35\text{-}36}$, *uvrA*$_{101\text{-}103}$, *dnaJ*$_{12\text{-}14}$, *sodB*$_{62\text{-}69}$, and *katE*$_{94\text{-}95}$.

Clones with the expected insert were immediately obtained for *dnaK*$_{101\text{-}103}$ and *katE*$_{94\text{-}95}$ (Fig. 19).

**Fig. 19 Gel electrophoresis of the clones obtained from cloning products of *dnaK*$_{101\text{-}103}$ and *katE*$_{94\text{-}95}$ in the pDrive cloning vector, digested with *Eco*RI.**



**Lanes 1-4** : PCR amplicon obtained with degenerate primer pair *katE*$_{94\text{-}95}$, cloned in pDrive, digested with *Eco*RI
**Lane 5** : 1kb DNA Ladder
**Lane 6-8** : PCR amplicon obtained with degenerate primer pair *dnaK*$_{101\text{-}103}$, cloned in pDrive, digested with *Eco*RI *dnaK* après digestion par *Eco*RI
**Lane 9** : 100 bp DNA Ladder

The inserts have been sequenced and alligned with the target genes (Table 4). Only at the site compatible with the degenerate primers sufficient complementarity was found. Translation of the nucleotide sequence in a protein sequence did not reveal the targetted stress related genes, i.e. *katE* encoding for Catalase in *R. rubrum* and *DnaK* for *Arthrospira* sp..

## *7.9. Discussion on the results of the identification of stress related genes using degenerated PCR in Arthrospira sp. and R. rubrum.*

The few results, obtained and the difficulties encountered in the optimisation of the method indicate that this method will not be able to produce positive results very fast. On the 24 known stress related genes only 17 had sufficiently conserved regions to enable the design of degenerate primer pairs. Only 11 PCR assays gave detectable bands after gel electrophoresis. After PCR optimisation it was observed that among those only 6 bands resided at the expected sequence length. Only two fragments were cloned for subsequent sequencing. Finally, it was observed after sequencing of the cloned fragments, that no targetted stress related gene was amplified using this method.

Redesigning of the primers, optimalisation of the PCR protocol and cloning of the remaining DNA amplicons could result in the identification of stress related genes in the target organisms.

On the other hand, the method was very simple in light of the techniques and hardware involved. It was seen as a first basis for identification of genes of interest for bacterial strains having an unknown genome. In the meantime *R. rubrum* ATCC11170 is being sequenced. Preliminary experiments indicate that *R. rubrum* ATCC25903 is very closely related to *R. rubrum* with a 99% sequence identity.

Nevertheless is the method very labour intensive, because it needs individual design of degenerate primers as well as PCR protocol optimisation. Furthermore, this method will be limited to a restricted number of stress related genes, that have already been identified in the scientific literature. Now that simultaneous detection and quantitative estimation of all transcripts in a micro-organism by DNA chip technology, and the technology of proteomic analysis by 2D proteomic mapping is available, these strategies may be more interesting to use.

Experimental work in this chapter was done by Florence Marty (SCK) during her final thesis.

Marty F. (2002) Identification de genes impliques dans la reponse au stress chez *Arthrospira platensis* and *Rhodospirillum rubrum*: Mise en place de stratégies. Mémoire en vue de l'obtention du titre d'ingénieur, Genie Biologique; CUST, Université Blaise Pascal, Clermont-Ferrand.

Sistrom W.R. (1960) A requirement for sodium in the growth of *Rhodopseudomonas spheroides*. J. gen. Microbiol. 22: 778-785.

Zarrouk C. (1966) Contribution à l'étude d'une cyanophycée. Influence de divers facteurs physiques et chimiques sur la croissance et la photosynthèse de *Spirulina maxima*. Thèse de Doctorat, Paris.

ANNEX 1:

Complete list of degenerate Primers designed for *Arthrospira* sp. PCC8005 and *R. rubrum* ATCC25903.

List of designed primers for *Arthrospira* sp.

| Gene | Primers | Sequence (5'-3') | Bases | Melting temperature (Tm) | Size (bp) | Degeneracy (%) |
|---|---|---|---|---|---|---|
| *dnaK* | 1 | GNATNGAYYTNGGNACNACC | 34-60 | 54.7 | 424 | 35 |
| | 2A | GCNGGHACNGTDATNACNGC | 439-458 | 61.3 | | 30 |
| | 1A | GGNATNGAYYTNGGNACNACC | 33-60 | 58.4 | 432 | 33.3 |
| | 2 | GGNNTCNNTNAANTANGCNGG | 439-465 | 50.6 | | 38.1 |
| | 3 | GGNATYGAYYTNGGWACNACC | 67-90 | 58.4 | 794 | 33.3 |
| | 6 | TTCRATTTTRGCYTTYTCSGC | 835-861 | 52.8 | | 23.8 |
| | 4 | CCNGCYTAYTTYAMYGAYYCC | 478-498 | 56.2 | 383 | 38 |
| | 6 | TTCRATTTTRGCYTTYTCSGC | 835-861 | 52.8 | | 23.8 |
| | 3 | GGNATYGAYYTNGGWACNACC | 67-90 | 58.4 | 431 | 33.3 |
| | 4 | GGRRTCRKTRAARTARGCNGG | 478-498 | 50.6 | | 38.1 |
| *dnaJ* | 12 | TTCGSAAGCTRGCWCGYCAG | 75-96 | 70 | 443 | 20 |
| | 14 | CCTCYARRRARCGRCCATCT | 499-519 | 65.5 | | 30 |
| | 14F | AAGATGGYCGYTYYYTRGAGG | 499-519 | 54.2 | 328 | 28.6 |
| | 15 | CACKAWYTGRTCWCCSCGATC | 807 | 62.7 | | 23.8 |
| | 14A | TGGAAGATGGYCGYTYYYTRGAGG | 496-519 | 61.8 | 331 | 25 |
| | 15A | CACKAWYTGRTCWCCSCGATCRCC | 804-827 | 72.2 | | 29.2 |
| *groEL* | 16A | AIGGNGTNATYWMVVTNGAA | 515-534 | 45.1 | 663 | 40 |
| | 16B | TCYTTSANTTCNRYTTCNGT | 1158-1178 | 42 | | 35 |
| | 16 | AANGANGGNGTNATYWMVVTNGAA | 511-534 | 53.4 | 353 | 41.6 |
| | 17 | CAYNKSYTTRCGVCGDTCRCCRAA | 841-864 | 68.5 | | 41.6 |
| | 18 | AHRYDGYYGGKGAYGGYACC | 245-264 | 55.8 | 371 | 40 |
| | 19 | GGTSRYRAAGTARGGVGARA | 596-615 | 50.8 | | 35 |
| | 19F | ATYTCBCCYTACTTYRYSACC | 595-615 | 47.8 | 548 | 33.3 |
| | 20 | TTTRATYACMGCYACACCMCC | 1123-1143 | 65.3 | | 23.8 |
| *groES* | 29 | CCYYTNGGCGATCGSGTKTTTGTG | 37-60 | 62.1 | 257 | 20.8 |
| | 31 | ATRRTCTTTYTCNGWNARCARNAC | 274-294 | 54.9 | | 37.5 |
| | 29 | CCYYTNGGCGATCGSGTKTTTGTG | 37-60 | 62.1 | 104 | 20.8 |
| | 30 | TTCRCCNAYTTGGGGYTTYTCYTT | 119-141 | 61.4 | | 25 |
| *clpB* | 35 | CCNGGNGTNGGNAARACNGCNATC | 1090-1113 | 69.8 | 389 | 29.1 |
| | 36 | GAANCKNCKNTCNAGNGCNGCRTC | 1446-1479 | 65.9 | | 37.5 |
| | 37 | CKGGKGTKGGYAAAACKGCG | 656-675 | 62.3 | 1660 | 25 |
| | 41 | CCGRTTSAGRAAYTCNGGNC | 2297-2316 | 60.4 | | 30 |
| | 39 | GGCCCYACWGGKGTNGGKAAA | 1858-1878 | 67.6 | 458 | 19 |
| | 41 | CCGRTTSAGRAAYTCNGGNCG | 2296-2316 | 64.7 | | 28.6 |
| *clpX* | 45 | ACCCTGACSGAAGCSGGCTAC | 523-542 | 58.9 | 719 | 9.5 |
| | 50 | AATAAATTCGGGAATCAGRCC | 1222-1242 | 58.4 | | 4.8 |
| | 45 | ACCCTGACSGAAGCSGGCTAC | 523-542 | 58.9 | 369 | 9.5 |
| | 47 | CAAAGCCSATSGATTTTTGC | 872-892 | 57.3 | | 9.5 |
| | 44 | GTMGCKGTTTAYAACCACTAC | 334-354 | 51.2 | 857 | 14.3 |
| | 49 | CCKMGCTCCGGTYTTACGRCG | 1171-1191 | 65 | | 19 |
| *regulonB* | 56 | GGKYTAGAACGRGGKGTMGAGAARTTT | 397-483 | 59.5 | 75 | 22.2 |
| | 57 | GTARGCATARGTGGARAACTTRTAYCC | 446-472 | 62.7 | | 18.5 |
| | 53 | ATYGGWCGKGTRCCYYTGTTA | 93-114 | 60.7 | 390 | 28.6 |
| | 56R | AAAYTTCTCKACMCCYCGTTC | 403-423 | 62.2 | | 19 |
| | 54 | ATGATTSARGCWAATCTSCGG | 301-321 | 62.6 | 171 | 19 |
| | 57 | GTARGCATARGTGGARAACTT | 452-472 | 52.7 | | 14.3 |
| | 53 | ATYGGWCGKGTRCCYYTGTTA | 93-114 | 60.7 | 279 | 28.6 |
| | 55 | CCGSAGATTWGCYTSAATCAT | 352-372 | 56.9 | | 19 |
| *sodB* | 62 | GCCTACGTYAAYAAYTWCAAC | 94-114 | 58.2 | 428 | 19 |
| | 69 | GGGACGACGGTTTTGRTAGTC | 508-528 | 56.3 | | 4.76 |
| | 62 | GCCTACGTYAAYAAYTWCAAC | 94-114 | 58.2 | 215 | 19 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 65 | GTCAGCGKYGATTTTGTCSGC | 295-315 | 57.9 | | 14.2 |
| | 62 | GCCTACGTYAAYAAYTWCAAC | 94-114 | 58.2 | 377 | 19 |
| | 67 | GGTCAGYARMGGRGTTTGACC | 457-477 | 58.9 | | 14.3 |
| *sodA* | 70 | TTYGGNWSNGGCTGGGYMTGG | 379-399 | 58.7 | 185 | 33.3 |
| | 72 | GTANTCYRRRCGKCKRTTYTG | 544-564 | 49.8 | | 42.9 |
| | 70 | TTYGGNWSNGGCTGGGYMTGG | 379-399 | 58.7 | 85 | 33.3 |
| | 71 | ATCARCGGRSWRRTCYTGRTT | 445-464 | 50.4 | | 38.1 |
| *katG* | 75 | ATGGRNYYNATYTAYGTNAMNCCSGAA | 681-707 | 61.1 | 587 | 40.7 |
| | 76 | CATRTCNCKGTNNRTCAGYTTRAACA | 1245-1268 | 59.7 | | 29.6 |
| | 83 | GTTAACCCKGARGGSSYGGAT | 748-768 | 55.1 | 1399 | 23.8 |
| | 91 | GGAGTTRGAACCAAACACCAG | 2128-2148 | 55.3 | | 4.7 |
| | 84 | GSCATGAAYGACGARGAAACC | 826-846 | 61 | 826 | 14.3 |
| | 87 | AGTCATTTCCGGYGCSGTYAG | 1894-1915 | 60.7 | | 14.3 |
| | 86 | CCSMARGAAGATYTRATYTGGCAA | 1357-1380 | 58.6 | 791 | 25 |
| | 91L24 | GGAGTTRGAACCAAACACCAGRTC | 2125-2148 | 58.5 | | 8.3 |
| | 86 | CCSMARGAAGATYTRATYTGGCAA | 1357-1380 | 58.6 | 664 | 25 |
| | 90 | CAARTTMACRAAGAAGTCRTTRCT | 1999-2022 | 56.3 | | 20.8 |
| | 90F | AGYAAYGACTTCTTYGTKAAYTTG | 1999-2022 | 55.5 | 149 | 20.8 |
| | 91 | GGAGTTRGAACCAAACACCAGRTC | 2125-2148 | 58.5 | | 8.3 |
| | 78 | CAAMSKTGGTGGCCMGCCGACTGG | 307-330 | 69 | 1688 | 16.7 |
| | 89 | TACKCCMACSCGRTCRGTRAAGAC | 1972-1995 | 67.2 | | 25 |
| *katA* | 92 | GAAAARYTNKCNCAYTTCRAC | 133-153 | 54 | 257 | 33.3 |
| | 93 | TTCNGTRTARAAYTTTNAYRGC | 370-390 | 49.4 | | 33.3 |
| *katE* | 94 | AARATSWYSCACTTYGAYCAYGAG | 364-387 | 61.1 | 290 | 33.3 |
| | 95 | GATRAAGAASAYNGGNRWGTTRTT | 631-654 | 54.1 | | 33.3 |
| *rpoS* | 98 | ATYCGCCAGASSATYGARCGG | 586-606 | 69 | 470 | 23.8 |
| | 100 | CTGGCGMACMCGYTCRCGSGT | 1036-1056 | 73.2 | | 23.8 |
| *uvrA* | 101 | ATYGGYYTNCAYCMNMGNGAYAAC | 1720-1743 | 62.9 | 583 | 37.5 |
| | 103 | AAANAYNCCKGTRTADGTNGCVGG | 2280-2303 | 62.7 | | 33.3 |
| | 105C | GGTCAGAAAAAGAWAATTCC | 1073-1092 | 51.8 | 1269 | 5 |
| | 105A | ACGTTGAAAGAAAATTGTCC | 2323-2342 | 50.7 | | 0 |
| | 105B | TCAGAAAACTTTGCCTGTCC | 889-908 | 54.1 | 1453 | 0 |
| | 105A | ACGTTGAAAGAAAATTGTCC | 2323-2342 | 50.7 | | 0 |
| *lexA* | 115A | ATGGAACSYCTMACMSRAGC | 1-20 | 55.2 | 189 | 30 |
| | 118 | GGCTTTRCCKTCAGTCCAWT | 170-189 | 56.4 | | 15 |
| *glnA* | 119 | AANATTNARMTKATYGACCTC | 46-66 | 50.5 | 1081 | 28.6 |
| | 122 | ATARGGRTTRGMSGTRGCRTC | 1111-1126 | 49.2 | | 33.3 |
| | 119 | AANATTNARMTKATYGACCTC | 46-66 | 50.5 | 995 | 28.6 |
| | 121 | ACGRTTWCCTTGGGARTARGC | 1021-1041 | 59.3 | | 19 |

## List of designed primers for *Rhodospirillum rubrum*

| Gene | Primers | Sequence (5'-3') | Bases | Melting temperature (Tm) | Size (bp) | Degeneracy (%) |
|---|---|---|---|---|---|---|
| *dnaK* | 1 | GNATNGAYYTNGGNACNACC | 34-60 | 54.7 | 424 | 35 |
| | 2A | GCNGGHACNGTDATNACNGC | 439-458 | 61.3 | | 30 |
| | 1A | GGNATNGAYYTNGGNACNACC | 33-60 | 58.4 | 432 | 33.3 |
| | 2 | GGNNTCNNTNAANTANGCNGG | 439-465 | 50.6 | | 38.1 |
| *dnaJ* | 8 | CGCARACYTGTCCGACCTGT | 512-531 | 61.3 | 528 | 10 |
| | 10 | AGGYYYACCGGKGTTTCGAC | 1021-1040 | 59.1 | | 20 |
| | 7 | TACGAGRTTYRGGSGTKTCC | 19-39 | 48.2 | 1021 | 23.8 |
| | 10 | AGGYYYACCGGKGTTTCGAC | 1021-1040 | 59.1 | | 10 |
| | 7 | TACGAGRTTYRGGSGTKTCC | 19-39 | 48.2 | 512 | 23.8 |
| | 8 | ACAGGTCGGACARGTYTGCGG | 512-531 | 62.6 | | 9.5 |
| *groEL* | 16A | AIGGNGTNATYWMVVTNGAA | 515-534 | 45.1 | 663 | 40 |
| | 16B | TCYTTSANTTCNRYTTCNGT | 1158-1178 | 42 | | 35 |
| | 16 | AANGANGGNGTNATYWMVVTNGAA | 511-534 | 53.4 | 353 | 41.6 |
| | 17 | CAYNKSYTTRCGVCGDTCRCCRAA | 841-864 | 68.5 | | 41.6 |
| | 24 | AAGGBATGCARTTCGACCGYGGYTACC | 572-598 | 69.1 | 319 | 14.8 |
| | 25 | GCCVGTCAGGRYYGCRAKRTCCTSCAG | 865-891 | 69.8 | | 29.6 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 24F | GBATGCARTTCGACCGYGGYTACC | 575-598 | 65.5 | 349 | 14.8 |
| | 26 | CTYCRTVCCGAKSTCKKCMGAGAT | 901-924 | 64.5 | | 33.3 |
| | 22 | TTCGARAAYRTGGGYGCSCAGATG | 196-219 | 68.6 | 157 | 20.8 |
| | 23 | CGYTTSAGRTCCATCGGRTTCATG | 330-353 | 61.2 | | 16.7 |
| *groES* | 32 | CCRYTGCATGAYCGYGTGMTSGTC | 13-36 | 64.7 | 264 | 25 |
| | 33 | CCAKRATGTCSSWYTCNKWCATGA | 254-277 | 57 | | 37.5 |
| *clpB* | 35 | CCNGGNGTNGGNAARACNGCNATC | 1090-1113 | 69.8 | 389 | 29.1 |
| | 36 | GAANCKNCKNTCNAGNGCNGCRTC | 1446-1479 | 65.9 | | 37.5 |
| *sodA* | 70 | TTYGGNWSNGGCTGGGYMTGG | 379-399 | 58.7 | 185 | 33.3 |
| | 72 | GTANTCYRRRCGKCKRTTYTG | 544-564 | 49.8 | | 42.9 |
| | 70 | TTYGGNWSNGGCTGGGYMTGG | 379-399 | 58.7 | 85 | 33.3 |
| | 71 | ATCARCGGRSWRRTCYTGRTT | 445-464 | 50.4 | | 38.1 |
| *katG* | 75 | ATGGRNYYNATYTAYGTNAMNCCSGAA | 681-707 | 61.1 | 587 | 40.7 |
| | 76 | CATRTCNCKGTNNRTCAGYTTRAACA | 1245-1268 | 59.7 | | 29.6 |
| *katA* | 92 | GAAAARYTNKCNCAYTTCRAC | 133-153 | 54 | 257 | 33.3 |
| | 93 | TTCNGTRTARAAYTTTNAYRGC | 370-390 | 49.4 | | 33.3 |
| *katE* | 94 | AARATSWYSCACTTYGAYCAYGAG | 364-387 | 61.1 | 290 | 33.3 |
| | 95 | GATRAAGAASAYNGGNRWGTTRTT | 631-654 | 54.1 | | 33.3 |
| *rpoS* | 98 | ATYCGCCAGASSATYGARCGG | 586-606 | 69 | 470 | 23.8 |
| | 100 | CTGGCGMACMCGYTCRCGSGT | 1036-1056 | 73.2 | | 23.8 |
| *uvrA* | 101 | ATYGGYYTNCAYCMNMGNGAYAAC | 1720-1743 | 62.9 | 583 | 37.5 |
| | 103 | AAANAYNCCKGTRTADGTNGCVGG | 2280-2303 | 62.7 | | 33.3 |
| *recA* | 107 | TTYGGYAARGGCTCSATCATG | 94-114 | 61.6 | 776 | 19 |
| | 114 | GCCSAGRTCRACSAGYTCGCC | 850-870 | 63.7 | | 23.8 |
| | 112 | TGATCTTCATCAACCAGATCC | 602-622 | 53.6 | 194 | 0 |
| | 113 | CGSNGCSAYYTTGTTCTTCAC | 775-796 | 54.5 | | 23.8 |
| | 107 | TTYGGYAARGGCTCSATCATG | 94-114 | 61.6 | 314 | 19 |
| | 110 | CTGYTCSCCSGTGTCSGGCTG | 388-408 | 63.1 | | 19 |
| | 108 | GGNAARACCACGCTGACSCTG | 316-336 | 68.4 | 244 | 14.3 |
| | 111 | TTSGGCGTCAGCCGCCACSGA | 469-491 | 69.9 | | 9.5 |
| | 108 | GGNAARACCACGCTGACSCTG | 316-336 | 68.4 | 623 | 14.3 |
| | 114 | GCCSAGRTCRACSAGYTCGCC | 850-870 | 63.7 | | 23.8 |
| | 108 | TCSGGNAARACCACGCTGACSCTG | 244-267 | 73.1 | 247 | 16.7 |
| | 111 | TTSGGCGTCAGCCGCCACSGAGTC | 466-491 | 71.9 | | 8.3 |
| | 107 | TTYGGYAARGGCTCSATCATG | 94-114 | 61.6 | 242 | 19 |
| | 109 | GTCNAGMGCRTGTTCRGCRTC | 316-336 | 55.6 | | 23.8 |
| | 107 | TTYGGYAARGGCTCSATCATG | 94-114 | 61.6 | 528 | 19 |
| | 112 | GGATCTGGTTGATGAAGATCA | 602-622 | 53.6 | | 0 |
| *glnA* | 124 | GAYGGCTCSTCSATYGSSGGC | 154-174 | 59.3 | 722 | 28.6 |
| | 129 | CAGRYCNGCRTATTTGTCSCC | 859-876 | 59.8 | | 23.8 |
| | 124A | AYGGCTCSTCSATYGSSGGC | 155-174 | 57.5 | 634 | 30 |
| | 127 | CATSGGYTTYGGCATRAAGG | 860-876 | 56 | | 20 |
| | 125 | GGYTACTTCCCGGTKMMMCCG | 538-558 | 60.5 | 339 | 23.8 |
| | 129 | CAGRYCNGCRTATTTGTCSCC | 859-876 | 59.8 | | 23.8 |
| | 124 | GAYGGCTCSTCSATYGSSGGC | 154-174 | 59.3 | 722 | 28.6 |
| | 131R | CGGAATACGGAYRSASSCMGA | 1027-1047 | 60.8 | | 28.5 |
| | 131 | TCKGSSTSYRTCCGTATTCCG | 1027-1047 | 50.7 | 140 | 28.6 |
| | 132 | GATCTTGTTCTTGATRCCRTC | 1147-1167 | 52.2 | | 9.5 |
| | 131 | TCKGSSTSYRTCCGTATTCCG | 1027-1047 | 50.7 | 191 | 28.6 |
| | 133 | TTCTTCYGGCGGCAGRTCRTA | 1198-1218 | 57.8 | | 14.3 |
| | 124 | GAYGGCTCSTCSATYGSSGGC | 154-174 | 59.3 | 488 | 28.6 |
| | 126 | TTCGTGGTGRTGSKYRTCNAC | 622-642 | 51.6 | | 28.6 |
| | 124 | GAYGGCTCSTCSATYGSSGGC | 154-174 | 59.3 | 404 | 28.6 |
| | 125 | CGGKKKMACCGGGAAGTARCC | 538-558 | 64.7 | | 28.6 |