Technical Note TN70.1

Part A: Literature, information and genomic databanks concerning the MELiSSA strains, closely related strains and the reference strain *R. metallidurans* CH34
State of the art and identification of critical points

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Abbreviations

ARDRA	amplified 16S and 16-23S ribosomal DNA gene sequences and restriction endonuclease analysis
ATCC	american type culture collection
BHR	broad host range
BSCW	basic support for cooperative work
DGGE	denaturing gradient gel electrophoresis
DNA	desoxyribonucleic acid
EMBL	european molecular biology laboratory
ERIC	enterobacterial repetitive intergenic consensus
IGS	intergenic spacers
ITS	internal transcribed spacers
IS	insertion sequence
kb	kilobase
Mb	megabase
MELiSSA	micro ecological life support system alternative
MG	malate glutamate (medium)
MOPS	2-N-morpholinepropanesulfonic acid
ORF	open reading frame
OROs	other relevant organisms
PCR	polymerase chain reaction
PPFD	photosynthetic photon flux density
rDNA	ribosmomal DNA
REP	repetitive extragenic palindromic
SMN	supplemented malate-ammonium medium
SRS	sequence retrieval system
SpTrEMBL	Swiss-prot TrEMBL
SWALL	non-redundant protein database
SWISS-PROT	Swiss-prot protein database
TrEMBL	translated EMBL database
TrEMBLnew	translated EMBL database updates
UV	ultraviolet

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1. The strains

1.1. Compartment 1 (C1): The liquifying compartment

1.1.1. The relevant strains

Under ideal conditions the liquefaction compartment should be colonized by a consortium of different but compatible bacteria. This mix of saccharolytic and proteolytic microorganisms enables the breakdown of various polymers present in the basic waste (predominantly feces). The crude protein concentration of feces is estimated to be 20-30% of the total dry weight. In order to have efficient cycling of N, S and C, an extensive degradation of proteins is required. Furthermore, this degradation should not form dead end products nor metabolites toxic to the phototrophic bacteria of the next compartment.

One of these bacteria is the thermophilic *Clostridia* species. *Clostridia* are non pathogenic anaerobic gram-positive bacteria that can grow at 60°C. The metabolic characteristics of the thermophilic *Clostridia* are adequate for the anaerobic degradation of polymers. Molecular community fingerprinting of amplified 16S rDNA by denaturing gradient gel electrophoresis (DGGE) revealed three other bacterial strains as possible dominant species in the first compartment. These are: *Ruminococcus bromii*; *Petrotoga mobilis* and the CDC group DF-3 (TN43.2).

Ruminococcus is an anaerobic, chemoorganoheterotrophic heterofermentative gram-positive bacterium. They are able to form acetic and formic acids from carbohydrates and many can use cellulose.

Petrotoga is an obligatory anaerobic thermophilic bacterium. The fermentative sheated gram-negative bacterium is capable of reducing elemental sulphur to hydrogen sulfide and tolerates high salt concentrations.

The CDC group DF-3 is related but different to *Capnocytophaga* species and constitutes a separate genus that clusters together with *Bacteroides forsythus* and *Bacteroides distasonis*. It is a rare isolate from blood, stools and wounds (TN43.2).

1.1.2. Genetic stability of the relevant strains

Events of horizontal gene transfer can possibly occur inside the first compartment. Nevertheless it is important to investigate the presence of plasmids inside the unknown consortium. Emphasis should be put on the presence of broad host range plasmid and plasmids carrying antibiotic resistance genes or virulence genes to investigate undesired gene transfer in the next compartments.

Secondly, it will be important to use strains which are capable to survive under space related stress conditions. Mutations caused by space related stress conditions or accumulated during long time culturing are definitely undesirable.

Clostridium acetobutylicum resists radiation as a mutagen (Bowring and Morris, 1985). Radiation could in this organism even be used as a means of inducing certain desirable genes constructed especially for controlled gene expression, both spatially and temporally (Nuyts et al., 2001). Until now no report exist on the effect of radiation on *Ruminococcus* or *Petrotoga* strains. *Bacteroides* strains seem to cope with UV radiation (Winter et al., 2001). However, *Bacteroides fragilis* reacts very fast on different types of stress. When the response to heat shock was investigated in the obligate anaerobe *Bacteroides fragilis*, the cells responded quickly to stress and synthesised seven heat shock proteins immediately upon exposure to heat. The apparent molecular weights of the seven proteins differed from the apparent molecular weights of the proteins induced

by UV irradiation, O_2 and H_2O_2. Heat shock did not induce phage reactivation whereas UV irradiation, O_2 and H_2O_2 did induce phage reactivation systems (Goodman et al., 1985). Hopefully *Bacteroides* strains residing in the first compartment do not share the latter characteristic considering the fact that unstable culturing as well as horizontal gene transfer needs to be avoided in the MELiSSA loop.

1.2. Compartment 2 (C2): Carbon transformation

1.2.1. *Rhodospirillum rubrum*

The second compartment includes the treatment of the low molecular C-compounds as well as CO_2, H_2 and H_2S. This will be accomplished by *R. rubrum*. *Rhodospirillum* are nonsulfur purple bacteria that can be found in stagnant water bodies, lakes, waste-water ponds, sewage treatment plants, coastal lagoons, sediment, moist soil, and paddy fields, growing best where there is a significant amount of soluble organic matter. *Rhodospirillum* can also be found in almost any anoxic environment that is exposed to sufficient light to allow photosynthesis (ATCC Connection, May 2001). Photoautotrophic growth is possible with molecular hydrogen as electron donor. Cells preferably grow photoheterotrophically under anaerobic conditions in the light with various organic compounds as carbon and electron sources (Trüper and Imhoff, 1996).

1.2.2. Horizontal gene transfer in *R. rubrum*

Very little is known about the existence of restriction-modification systems in *Rhodospirillum*. *Rhodospirillum rubrum* is very accessible to BHR plasmids (Olsen and Shipley, 1973; Saegesser et al., 1992) and to conjugative plasmids that were currently used to introduce transposons in this strain (Bao et al., 1991; Jiang et al., 1998). Among the MELiSSA strains, *R. rubrum* is certainly the strain that looks the most permeable to plasmid-mediated gene dissemination. This feature is also enhanced by the crucial position of *R. rubrum* in the second compartment, just downstream of the first compartment containing the unknown consortium. Several different strains of *R. rubrum* contain a 55 kb plasmid (Kuhl et al., 1983; Kuhl et al. 1984). The curing of this plasmid irreversibly damaged the capacity to grow photosynthetically and the production of pigment. The plasmid has likely a narrow host range and no information about its transfer capabilities is directly available (TN70.7).

No phage was reported for *R. rubrum*, although a rhizobiophage may integrate in the chromosome of *R. rubrum* in a tRNA gene (TN70.7). It is needed to check for the presence of defective prophage, which is able to constitutively transduce bacterial DNA fragments in *R. rubrum* because it was found in a related bacterium, *Rhodobacter capsulatus*. Likewise the possibility to produce anti viral or anti-microbial substances should be investigated which could be detrimental to optimal reactor conditions (Suwanto and Kaplan, 1991; Guest, 1974) (TN70.7).

1.3. Compartment 3 (C3): Nitrogen transformation

1.3.1. *Nitrosomonas europea* and *Nitrobacter winogradskyi*

The third compartment would receive mineralized products from compartment 2 containing ammonium, sulfate and phosphates. The main function of compartment 3 would be to recycle ammonium. Ammonium is processed into nitrate through nitrite by the nitrifying bacteria *Nitrobacter* and *Nitrosomonas* respectively. Carbondioxide is used as the carbon source.

1.3.2. Horizontal gene transfer in *N. europea* and *N. wynogradski*

There is up to now only one report that describes the presence of plasmids in one strain of *Nitrosomonas* (Yamagata et al., 1999). Recently a bioluminescent *Nitrosomonas* was constructed via conjugative transfer, thereby establishing conjugation as a tool for gene transfer into *Nitrosomonas* strains (Ludwig et al., 1999).

There is no report about the presence of phages in *Nitrosomonas*. However, phage-like bodies were reported in a series of German papers for *Nitrobacter* (Bock, 1974; Bock, 1976; Peters et al., 1974; Westphal and Bock, 1974).

Practically nothing is known about the existence of restriction-modification systems in *Nitrosomonas*.

1.4. Compartment 4 (C4): Food and Oxygen production

1.4.1. *Arthrospira platensis*

Arthrospira is an inexpensive, high quality nutritional supplement (Ciferri and Tiboni, 1985). The organism lives in warm lakes with high carbonate content and high pH. *Arthrospira* belongs to the cyanobacteria, a phylogenetically coherent group of evolutionarily ancient, morphologically diverse, and ecologically important phototrophic bacteria. The cyanobacteria are defined by the ability to carry out oxygenic photosynthesis. They all synthesize chlorophyll a as photosynthetic pigment, and most types synthesize phycobiliproteins as light-harvesting pigments. All cyanobacteria are able to grow using CO2 as the sole source of carbon (Lederberg, 2000).

Cyanobacteria are a source of structurally diverse polysaccharides. Environmental conditions and composition of the feed will have an effect on productivity and chemical composition of *Spirulina* (Olguín, 2000).

1.4.2. Horizontal gene transfer in *A. platensis*

Cyanobacteria may contain conjugative plasmids (Billi et al., 2001). They are accessible to BHR plasmids that were used to introduce vectors or transposons, but restriction is clearly an important barrier limiting the access of foreign DNA (Wok et al., 1984; Kreps et al., 1990; Sode et al., 1992; Marraccini et al., 1993; Ren et al., 1998; Elhal et al., 1997). Evidence has been put forward that a *Synechocystis* strain contains IS-elements, possibly spread through horizontal gene transfer between evolutionary distant organisms (Cassier-Chauvat et al., 1997).

The cyanobacterium *Synechocystis* sp. PCC 6803 is transformable at high efficiency and integrates DNA by homologous double recombination (Grigorieva and Shetstakov, 1982; Williams, 1988; Kufryk et al., 2002).

Cyanophages were found in marine *Synechococcus* (Fuller et al., 1998; Lu et al., 2001), filamentous heterocystous *Anabeana* and *Nostoc* strains (Bancroft and Smith, 1988), and LPP strains (*Lyngbya-Phormidium-Plectonema*). Mass lytic processes were also observed in microbial communities colonised by filamentous cyanobacteria (van Haanen et al., 1999). Lysogeny has been observed in a marine *Synechococcus* (McDaniel et al., 2002). This may be important as the lysogenic mode of life (phage genomes remain silent in the hosts genome) may revert to the lytic mode of life (activation of the phage genes, phage synthesis and cell lysis) under induction of UV light or other radiations (TN70.7).

A novel mechanism of site-specific recombination in the cyanobacterium *Synechococcus* sp. PCC7002 was discovered (Akiyama et al., 1998). The authors found a palindromic element for which the core element functioned as a resolution site for site-specific plasmid recombination. Although this element has not been detected in *Arthropspira platensis*, it was over-represented in the plasmid and in the genome of PCC7002, *Synechococcus* strains PCC6301, PCC7942, *vulcanus* and *Synechocystis* sp. PCC6803, suggesting that the site-specific recombination mechanism based on the palindromic element could be common in cyanobacteria (Akiyama et al., 1998).

1.5. The reference strain *R. metallidurans* CH34

1.5.1. *Ralstonia metallidurans*

Ralstonia metallidurans CH34 (ATCC43123) is a unicellular non-sporeforming Gram-negative bacterium. CH34 possesses an oxidative metabolism and she can use a large array of C-sources. CH34 is a facultative chemolithotrophic organism and can therefore use H_2 as an energy source and CO_2 as a carbon source. In the presence of nitrate *R. metallidurans* CH34 can even grow anaerobic (Taghavi, 1996).

CH34 possesses two endogenous megaplasmids, pMOL28 (180 kb) and pMOL30 (240 kb), which provide their host with resistance against Co^{2+}, Ni^{2+}, CrO_4^{2-}, Hg^{2+}, Pb^{2+}, Cd^{2+}, Cu^{2+} and Zn^{2+} (Collard et al., 1994).

1.5.2. Genetic stability of *R. metallidurans*

CH34 is an excellent host for acquisition and expression of foreign genes. She is very amenable for genetic manipulation via conjugation (Springael et al., 1994). The strain can be used to select novel BHR plasmids directly from environmental samples by triparental exogenous isolation (Top et al., 1994).

It is important to mention that a certain mutagenesis phenomenon, dependent on temperature, was discovered with CH34. The mutation phenomenon was called temperature induced mutagenesis and mortality (Taghavi et al., 1997). Investigations on *R. metallidurans* and related organisms report that other stress factors like gene transfer, storage in liquid nitrogen and plasmid incompatibility can induce mutagenesis next to the known effects of chemical and physical mutagens.

2. Culturing media of *R. rubrum*

2.1. Rich media

2.1.1. ATCC Culture Medium 112 Van Niel's yeast agar (Ronald M. Atlas, 1993 (Handbook of Microbiological Media))

(Use : for the cultivation and maintenance of Rhodobacter sphaeroïdes)

K_2HPO_4	1g
$MgSO_4$	0.5g
Yeast extract	10.0g
Agar	20.0g
Tap water	1 liter

PH 7.0-7.2

Temperature : 26°C
Anaerobic growth condition

2.1.2. ATCC Culture Medium 1308 *Rhodospirillum medium* (Ronald M. Atlas, 1993 (Handbook of Microbiological Media))

(Use : for the cultivation and maintenance of Rhodospirillum species)

=> Culture not proved

Yeast extract,	1.0g
Ethanol,	0.5ml
Disodium succinate,	1.0g
0.1% Ferric citrate (aqueous),	5.0ml
KH_2PO_4,	0.5g
$MgSO_4 . 7H_2O$,	0.4g
NaCl,	0.4g
NH_4Cl,	0.4g
$CaCl_2 . 2H_2O$,	0.05g
Trace Elements Solution SL-6 (see below),	1.0ml
Sodium ascorbate,	0.5g
Distilled water,	1.0 Liter

Final pH 6.0. Autoclave at 121°C, 15 minutes.

Trace Elements Solution SL-6:

$ZnSO_4 . 7H_2O$,	0.10g
$MnCl_2 . 4H_2O$,	0.03g
H_3BO_3,	0.3g
$CoCl_2 . 6H_2O$,	0.2g
$CuCl_2 . 2H_2O$,	0.01g
$NiCl_2 . 6H_2O$,	0.02g
$Na_2MoO_4 .H_2O$,	0.03g
Distilled water,	1.0 Liter

Adjust final pH of Trace Elements Solution SL-6 to 3.4

Temperature : 30°C

Growth condition : anaerobic

2.1.3. ATCC Culture Medium 1408 *Rhodospirillum rubrum* (Ronald M. Atlas, 1993 (Handbook of Microbiological Media))

(Use : for the cultivation of Rhodospirillum species)

=> Culture not proved

NaCl,	10.0g
$MgCl_2 . 6H_2O$,	3.5g
Yeast extract,	1.5g
Peptone,	1.5g
Sodium malate,	1.4g
KH_2PO_4,	0.3g
SLA Trace Elements (see below),	1.0ml
Distilled water to,	1.0L

Adjust medium for final pH 7.0. Autoclave at 121°C for 15 minutes.

SLA Trace Elements:

$CoCl_2 . 6H_2O$,	250.0mg
$NiCl_2 . 6H_2O$,	10.0mg
$CuCl_2 . 2H_2O$,	10.0mg
$MnCl_2 . 4H_2O$,	70.0mg
$ZnCl_2$,	100.0mg
H_3BO_3,	500.0mg
$Na_2MoO_4 . 2H_2O$,	30.0mg
$Na_2SeO_3 . 5H_2O$,	10.0mg
$FeCl_2 . 4H_2O$,	1.8g
Distilled water,	1.0 L

Adjust trace element solution to pH 2-3

Temperature : 26°C

Growth condition : anaerobic

2.1.4. R8AH medium *Rhodospirillum rubrum* (Ronald M. Atlas, 1993 (Handbook of Microbiological Media))

(Use : for the cultivation and maintenance of Rhodobacter sphaeroides, Rhodocyclus tenuis, Rhodopseudomonas rutila, Rhodospirillum photometricum, and Rhodospirillum rubrum)

Malic acid,	2.5g
Yeast Extract (Difco 0127),	1.0g
$(NH_4)2SO_4$,	1.25g
$MgSO_4 . 7H_2O$,	0.2g
$CaCl_2 . 2H_2O$,	0.07g
Ferric citrate,	0.01g
EDTA,	0.02g
KH_2PO_4,	0.6g
K_2HPO_4,	0.9g
Trace Elements (see below),	1.0ml
Vitamin Solution (see below),	7.5ml
Distilled water to,	1.0Liter

Neutralize malic acid with NaOH and adjust the pH of the completed medium to 6.9. Autoclave at 121°C for 15 min.

Trace Elements :

Ferric citrate,	0.3g
$MnSO_4 . H_2O$,	0.002g
H_3BO_3,	0.001g
$CuSO_4 . 5H_2O$,	0.001g
$(NH_4)_6Mo_7O_{24} . 4H_2O$,	0.002g
$ZnSO_4$,	0.001g
EDTA,	0.05g
$CaCl_2 . 2H_2O$,	0.02g
Distilled water,	100.0ml

Vitamin Solution :

Nicotinic acid,	0.2g
Nicotinamide,	0.2g
Thiamine . HCl,	0.4g
Biotin,	0.008g
Distilled water,	1.0Liter

Temperature : 30°C
Growth condition : anaerobic under a tungsten lamp (or 60W incandescent bulb)

2.1.5. SMN medium (Supplemented Malate-Ammonium medium; rich medium for *R.rubrum*)
=> For cultivation

- Culture in liquid medium

R.rubrum is cultivated at 30°C in SMN medium which is a modification of Ormerod medium supplemented with 0.3% casein enzyme hydrolysate and 0.3% Difco yeast extract (Fitzmaurice *et al.,*1989).
For phototrophic growth, cells are cultivated in 100-ml serum vials (average volume, 121 ml) containing 15 ml of N_2-sparged medium under an N_2 headspace. Vials are sealed with butyl rubber stoppers before autoclaving. Vials are agitated (2.5-cm throw, 290 rpm) to ensure uniform exposure of the cultures to the headspace gases and the tungsten light illumination (intensity, ca. 825 microeinsteins m-2 s-1) (Kerby *et al*, 1992).
Aerobic growth in the dark is conducted in 500-ml flasks containing 50 ml of medium; the flasks are agitated at 270 rpm (2.5-cm throw). Growth temperature is maintained at 30°C (Grunwald *et al*., 1994)
=> Liquid cultures are grown in SMN medium photoheterotrophically in screw-top tubes or aerobically with shaking.

- Culture on solid medium

R. rubrum is routinely grown on SMN medium plates photoheterotrophically in GasPak jars with tungsten illumination or aerobically (Shelver *et al*, 1995)**.**

☞**Specific anaerobic dark growth on carbon monoxide**

Phototrophic anaerobes such as *Rhodospirillum rubrum* have the ability to use CO as the sole carbon source and energy source. This ability derives from the oxidation of CO to CO_2 catalysed by carbon monoxyde desydrogenases (CODHs).
R. rubrum can grow quickly in liquid and plate CO- and Ni-dependent dark anaerobic cultures.
Liquid cultures are cultivated in RRNCO medium containing (per liter of distilled tap water)
2 µg of biotin, 10 ml of a chelated iron-molybdenum solution (0.28 g of H_3BO_3, 2 g of Na_2EDTA, 0.4 g of ferric citrate, and 0.1g of Na_2MoO_4 per liter of glass-distilled water),
250 mg of $MgSO_4.7H_2O$,132 mg of $CaCl_2.2H_2O$, 1g of NH_4Cl, 20 µM $NiCl_2$, 1.0 g of yeast extract, 2.1 g of morpholinopropanesulfonic acid (MOPS), and 0.82 g of sodium acetate as a non fermentable carbon source.

The medium, pH ajusted to 7.1, is prepared under strictly anaerobic conditions and dispensed under Ar (rendered oxygen free by passage through heated copper filings) to a volume of 10 ml per 100-ml serum vial (average volume, 121 ml).
Vials are sealed with butyl rubber stoppers prior to autoclaving. Anaerobic solutions of 0.05 ml of 1.91 M potassium phosphate (pH 7.0), 0.1 of 1% $Na_2S.9H_20$, 0.25 ml of 0.5 M $NaHCO_3$ (pH 8.0), and filter-sterilized CO are added prior to inoculation. Vials are incubated horizontally on a reciprocal shaker (90 oscillations per min; 2.5-cm throw) at 30°C in the dark for CO-dependent growth.

The plate medium is agar-solidified (1.2%) SMN medium supplemented with phosphate buffer (5ml/liter of medium) and $NiCl_2.6H_2O$ to the indicated levels. Plates (100 by 15 mm), which contained 30 ml of medium, are prepared and inoculated aerobically and then are incubated overnight in the dark in GasPak jars under an H_2-CO_2 atmosphere prior to introduction of CO to approximately 40% (Kerby *et al,* 1995**).**

2.2. Minimum media

2.2.1. MELISSA medium *Rhodospirillum rubrum (Segers and Verstraete, 1983)!*

(Minimum culture medium Rhodospirillum rubrum)

=> For axenic cultivation

Macro-elements solution (concentration g/l)

NH_4Cl	0.76
NA_2SO_4	0.54
EDTA	0.02
$MnCl_2$, $4H_2O$	0.01
CH3COOH	2.5
$MgSO_4$, $7H_2O$	0.2
$CaCl_2$, $2H_2O$	0.05
KH_2PO_4	0.49
K_2HPO_4	0.52
$FeSO_4$, $7H_2O$	0.02
$NaHCO_3$	0.25
MOPS	21

Trace elements (concentration g/l)

$NiSO_4$, $6H_2O$	0.5
$ZnSO_4$, $7H_2O$	0.1
$CuSO_4$, $5H_2O$	0.005
H_3BO_3	0.1
Na_2MoO, $2H_2O$	0.05

Vitamin (concentration g/l)

Biotine	0.015

1 ml of trace elements solution is added per 1 liter of macro-elements solution.
Final pH of the medium

Temperature : 30°C
Anaerobic condition
Fluorescent tubes (PHILIPS ® TLD 30W/83) provides the light on one side, with a light flux of about 20W/m2 (Gauthey T., 2001).

2.2.2. Ormerod medium (Bose *et al*, 1961)

=> For cultivation

Original formulation medium

KH_2PO_4 :	600 mg
K_2HPO_4 :	900 mg
$MgSO_4.7H_2O$:	200 mg
Mn $SO_4.4H_2O$:	210 mg
$Na_2MoO_4.2H_2O$:	75 mg
$FeSO_4.7H_2O$:	11.8 mg
$ZnSO_4.7H_2O$:	11.8 mg
Trace element solution :	1 ml
EDTA :	20 mg
Biotin :	15 µg
DL-malic acid :	6g
$(NH_4)_2SO_4$: 1.25g or DL- glutamic acid :	2g

Trace Element Solution :
(containing per 100 ml of deionised water)

H_3BO_3 :	280 mg
Mn $SO_4.4H_2O$:	210 mg
$Na_2MoO_4.2H_2O$:	75 mg
$ZnSO_4.7H_2O$:	24 mg
$Cu(NO_3)_2 .3H_2O$:	4 mg

The pH of the medium is adjusted to 6.8 with NaOH before autoclaving.

Growth conditions :

Stock liquid cultures are grown photo synthetically in 15 ml screw-cap test tubes, completely filled with a medium similar to that specified above, but containing 2 g of DL-malic acid and 0.5 g of $(NH_4)_2SO_4$ per liter; the cultures are transferred every 24 hours with 10% inoculum. A 1% inoculum of 24-hour-old cells is used to initiate growth in larger scale cultures in Roux bottles (or Erlenmeyer flasks) with 5% CO_2 in helium as the gas phase. The latter cultures are illuminated (Lumiline bulbs; light intensity, 600 foot-candles) at a temperature of approximately 25°C (Bose *et al*, 1961).

Ormerod medium as modified by Kanemeto & Ludden

Ormerod medium has got the following modifications (Kanemeto *et al*., 1984) : L-glutamate (as the nitrogen source) is increased to 27 mM, potassium phosphate is reduced to 0.37mM, and 52 mM (2-N-morpholinepropanesulfonic acid) (MOPS) is added. The medium is adjusted to pH 6.7 with NaOH before autoclaving. Cell cultures are grown at 30°C in water-jacketed fermentor vessels (150 to 500 ml) with filter-sterilized helium gas blown over the culture.
Illumination is provided by a 150-W reflector flood lamp located 4 cm from the vessel.

2.2.3. Malate-Glutamate Medium

=> Growth medium for the study of the nitrogenase system (minimal medium before nitrogenase derepression medium)

R.rubrum is grown first in rich (SMN) medium, then directly inoculated into Malate-Glutamate (MG) medium with 60-fold dilution.
The MG medium is a minimal medium where *R. rubrum* grows anaerobically and photosynthetically (Lehman, 1991).
This minimal medium contains the following (per liter) : 10.5g of MOPS (morpholinopropanesulfonic acid), 4g of malic acid, 1g of NH_4Cl, 2.8 mg of H_3BO_4, 20 mg of disodium EDTA, 4 mg of ferric citrate, 1 mg of Na_2MoO_4, 600 mg of KH_2PO_4, 900 mg of K_2HPO_4, 250 mg of $MgSO_4$, 100 mg of $CaCl_2$, and 1 µg of biotin (pH 7.0).

Note : Nitrogenase derepression medium used is the MG minimal medium with 4g of glutamic acid substituted for NH_4Cl and 0.75 g instead of 10.5 g of MOPS per liter. Cultures were derepressed by diluting minimal medium-grown cells 1:50 into derepression medium.

The cultures are led in anaerobe tubes with black butyl rubber stoppers and aluminium crimps, flushing the headspace with Ar, and incubating cultures anaerobically with illumination of 25 W/m^2 at 28°C .
The MG medium was supplemented with fructose (5 $g.l^{-1}$) to increase cell yields.
Some cultures are carried out under illuminated anaerobic conditions in serum bottles with the headspace flushed with oxygen-free nitrogen gas. After inoculation with 2% inoculum, all tubes are placed in darkness for a 24-h period to allow any residual oxygen to be used, thus avoiding possible photo-oxidative damage to cells when they are placed at 30°C under 5,000 lux of incandescent illumination (Mc Grath *et al.*, 1997).

2.2.4. Sistrom medium supplemented with cystine

Formulation of the Sistrom medium
(SISTROM, W.R., 1990)

Stock solution	Concentration per liter
Solution C*	20 ml
Potassium phosphate (pH 6.8)	20 ml
$(NH)_4SO_4$, 10% (w/v)	5 ml
Potassium succinate, 10%	20 ml
L-Glutamic acid, 5%	2 ml
L-Aspartic acid, 2%	2 ml

Solution C* composition:	
Nitrilotriacetic acid	10.0 g
$MgSO_4$. $7H_2O$	29.5 g
$CaCl_2.2H_2O$	3.335 g
$FeSO_4.7H_2O$	99.0 mg
$(NH_4)_6Mo_7O_{24}$. $4H_2O$	9.25 mg

Nicotinic acid	50.0 mg
Thiamine HCl	25.0 mg
Biotin	0.5 mg
Trace elements solution	50.0 ml

The trace element solution contains in 100 ml:

$ZnSO_4$. $7H_2O$	1.095 g
Ethylenediamine tetraacetic acid	250 mg
$FeSO_4$. $7H_2O$	500 mg
$MnSO_4$. H_2O	154 mg
$CuSO_4.5H_2O$	39.2 mg
CO $(NO_3)_2$. $6H_2O$	24.8 mg
H_3BO_4	11.4 mg

The pH is ajusted to 6.8-7.0 with KOH for solution C.

2.2.5. Pfennig medium

(MELISSA, ESTEC/Contract 8 125/88/NL/FGCCN4, Technical Note I6)

Culture have been carried out on *Rhodospirillum rubrum* ATCC 1117
The medium of Pfennig has been used as a standard medium described in 'The Prokaryotes'(1981) with some adaptations.

Medium:	Conc. per liter
- Solution 1: 0.22% salts	
KH_2PO_4	0.34g
NH_4Cl	0.34g
KCl	0.34g
$MgSO_4.7H_2O$	0.5g
$CaCl_2.2H_2O$	0.25g
- Solution 2: trace elements	1 ml
- Solution 3: 0.002 % vitamin B12	1 ml
- Solution 4: 7.5 % $NaHCO_3$	20 ml
- Solution 5: A. 10 % $Na_2S.9H_2O$	4 ml
B. 3 % $Na_2S.9H_2O$	20 ml
- Solution 6: 5 % Mg/NH_4 acetate	10 ml

Solution 2: trace elements	Conc. per liter
- EDTA-di Na	3 g
- $FeSO_4.7H_2O$	1.1 g
- $CoCl_2.6H_2O$	190 mg
- $MnCl_2.2H_2O$	50 mg
- $ZnCl_2$	42 mg
- $NiCl_2.6H_2O$	24 mg
- $Na_2MoO_4.2H_2O$	18 mg
- H_3BO_3	300 mg
- $CuCl_2.2H_2O$	2 mg

Solution 6: Mg/NH_4 acetate	Conc. per liter
- $(CH_3COO)_2Mg$	25 g
- CH_3COONH_4	25 g

To prepare the medium, solutions 1 and 2 are brought together and autoclaved. The other 4 solutions are each individually sterilized. The vitamin B12 solution and the Na-bicarbonate solution are sterilized through a membrane filter. This prevents the destruction of the vitamin B12 at the high temperatures reached during autoclavation and the loss of Na-bicarbonate caused by gas formation (CO_2). The right quantities of solutions 3 and 4 are mixed with the autoclaved solutions 1 and 2, after cooling down.
The solutions 5a and 5b are prepared in bottles with rubberized plugs and then autoclaved. Also solution 6 is autoclaved. The appropriate quantities of the solutions 5 and 6 are added to the previous solutions. The whole is put at the ideal growth pH 7.3 (7-7.5).
The solutions 5b and 6 are supplementary solutions which could be added regularly during the growth of cultures.

The reason to sterilize all the different solutions individually is to avoid too much precipitation. When the solution is ready, we pour it in sterilized bottles or into test tubes closed with a plug.
When necessary to work with agar plates, 1.5 to 2% agar is added to the solution, composed to solutions 1 and 2; before autoclavation. The other solutions are added after sterilization, the whole is mixed and the plates are poured.

The ideal growth temperature for these microorganisms is about 30°C. This temperature determines which compounds will be soluble. The concentration of H_2S which is maximal soluble at 30°C is 3 g/l, for HS^- it is 6 g/l, for CO_2 1.3 g/l and for HCO_3^- 99 g/l. The solubility degree of CH_3COO^- and NH_4^+ in the medium is of less importance because their Ks values are very high. H_2 has a very low solubility degree, at 30°C 1.5 mg/l.

When the bacteria are grown under phototrophic conditions, 'Sylvania Gro Lux' lightenings are chosen as light source. These lightenings have an emission area between 400-500 and 600-700 nm, which corresponds with the absorption area of the bacteriochlorophyll a and the carotenoids (spirilloxanthin series).

3. Culturing media of *Arthrospira platensis*

3.1. Minimal medium

3.1.1. ZARROUK medium for *Arthrospira platensis* (Zarrouk, 1966)!

(SOT medium : for the cultivation and maintenance of *Spirulina maxima* and *Spirulina platensis*) (Ronald M. Atlas, 1993)

Macro elements solution (concentration in $g.l^{-1}$)

- $NaNO_3$:	2.5
- K_2SO_4 :	1
- NaCl :	1
- $MgSO_4, 7H_2O$:	0.2

- $CaCl_2, 2H_2O$: 0.04
- $FeSO_4, 7H_2O$: 0.01
- EDTA $Na_2, 2H_2O$: 0.08
- K_2HPO_4 : 0.5
- $NaHCO_3$: 10.8
- Na_2CO_3 : 7.6

+ 1 ml per liter of each metallic solution

The various salts of the macro elements should be introduced into the solution in the written order. Phosphate should always be added last.

Metallic solution (concentration in $g.l^{-1}$)
Solution A5 :

- H_3BO_3 : 2.86
- $MnCl_2, 4H_2O$: 1.81
- $ZnSO_4, 7H_2O$: 0.222
- $CuSO_4, 5H_2O$: 0.079
- MoO_3 : 0.015

Solution B6 :

- NH_4VO_3 : 0.023
- $KCr(SO_4)_2, 12H_2O$: 0.096
- $NiSO_4, 7H_2O$: 0.048
- $(NO_3)_2Co, 6H_2O$: 0.049
- $Na_2TnO_4, 2H_2O$: 0.018
- $Ti(SO_4)_2 + TiOSO_4$: 0.048

The freshly prepared solution should have a pH in the range 8.7 to 9.3
Because of the poor solubility of NH4VO3, B6 solution tends to be turbid. This solution should be well stirred before usage.
Solutions A5 and B6 should be kept refrigered, replacing them after 2 months

Growth conditions

(Vonshak, 1997)

Growth temperature : 35°C

Arthrospira may be easily photo inhibited, thus one should make sure to start the culture in dim light, i.e. 15 µE, and gradually increase irradiance.
Once the culture begins to grow, pH should be kept at about 9.8 by bubbling CO2 ca. 1-2 per cent in air. When grown in tubular (3 cm diameter) vessels, the initial chlorophyll concentration should be 1-2 $mg.ml^{-1}$.
A growth rate curve should be followed, and the best cell density in which to maintain the culture is at ca. ½ µmax.

Strain can be grown in batch cultures on an orbital shaker at 30°C under continuous cool white illumination providing a photon flux density of 100 µEm-^{2}.s^{-1} (VITI *et al*., 1997).
Cultivation can be also performed in glass containers subjected to a moderate mixing provided by a small air pump operation at a rate of 0.046 vvm (volumetric flow rate of air per volume of liquid per minute).
The depth of the liquid column is always 0.10 m. Cultures are exposed to tungsten lamps (39 W), to provide either 66 or 144 µmol photon . m^{-2}.s^{-1}, depending on the kind of experiment in which they were used (OLGUIN *et al*., 2001).
Some cultures of *Spirulina platensis* are also made at photon flux density (PPFD) of 50 µmol.m^{-2}. s^{-1} provided by fluorescent lamps at 35°C (LU, 2000).

Maintenance

Cultures can be maintained on solidified medium (1.2-1.5 per cent agar). If kept at low light of 10-20 µmol.m^{-2}.s^{-1} and 20°C, cells will be viable for more than 6 months if not heavily contaminated by bacteria.

3.1.2. *Spirulina* medium (Schlösser, 1982)[1]

=> For cultivation

Modification of the SAG medium. Suitable for LB 2340 *Spirulina platensis* and LB 2342 *Spirulina maxima*.

Solution A

Glass-distilled water	500 mL
$NaHCO_3$	13.61 g
Na_2CO_3	4.03 g
K_2HPO_4	0.5 g

Solution B

Glass-distilled water	500 mL
$NaNO_3$	2.5 g
K_2SO_4	1.00 g
NaCl	1.00 g
$MgSO_4.7H_2O$	0.2 g
$CaCl_2.2H_2O$	0.04 g

Solution A and B are autoclaved separately in order to prevent the formation of precipitates and combined aseptically after cooling.

PIV metal solution	6 mL
Chu micronutrient solution	1 mL
Vitamin B12 (15µg/100 mL H2O)	1 mL

PIV metal solution

To 1000 mL of glass-distilled water, add 0.750 g of Na_2EDTA, and dissolve fully.

[1] http://www.bio.utexas.edu/research/utex/media/spirulina.html

$FeCl_3.6\ H_2O$	97 mg
$MnCl_2.4H_2O$	41 mg
$ZnCl_2$	5 mg
$CoCl_2.6H_2O$	2 mg
$Na_2MoO_4.2H_2O$	4 mg

Chu micronutrient solution
To 1000 mL of autoclaved glass-distilled water

Na_2EDTA	50.0 mg
H_3BO_3	618.0 mg
$CuSO_4.5H_2O$	19.6 mg
$ZnSO_4.7\ H_2O$	44.0 mg
$CoCl_2.6\ H_2O$	20.0 mg
$MnCl_2.4\ H_2O$	12.6 mg
$Na_2MoO_4.2H_2O$	12.6 mg

Growth condition

Strains are cultivated in *Spirulina* medium under low light (10 to 40 $\mu E.m^{-2}.s^{-1}$) at a constant temperature of 25°C (Scheldeman *et al.*, 1999).

3.1.3. ASN-III medium[2]

=> For maintenance

NaCl	25.0
$MgCl_2.6H_2O$	2.0
KCl	0.5
$NaNO_3$	0.75
$K_2HPO_4.3H_2O$	0.02
$MgSO_4.7H_2O$	3.5
$CaCl_2.2H_2O$	0.5
Citric acid	0.003
Ferric ammonium citrate	0.003
EDTA (disodium magnesium)	0.0005
Na_2CO_3	0.02
Trace metal mix A5 + Co	1 mL
Deionized water	to 1 liter

PH after autoclaving and cooling : 7.5

Trace metals A5 + Co

H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.222
$Na_2MoO_4.2H_2O$	0.390
$CuSO_4.5H_2O$	0.079
$Co(NO_3)_2.6H_2O$	0.049

[2]http :// www.pasteur.fr/recherche/banques/PCC/Media.htm

Growth condition

Strains are maintained at 25 °C at a photosynthetic photon flux density of approximately 5 µmol m^{-2} sec^{-1} and a light regime of 12 h light / 12 h dark.

3.1.4. ES-enriched seawater medium[3]

(Provasoli, 1963)

⇨ General purpose marine medium for axenic cultures

Preparation : Add 1 tube (20 ml) of ES-enrichment to 1000 ml of pasteurized, filtered seawater. For ES-enrichment solution, add the following to 1000 ml glass-distilled water :

$NaNO_3$	350mg
Na_2glycerophosphate.$5H_2O$	50mg
Fe-solution	25 ml
PII metals	25 ml
Vitamin B12	10µg
Thiamine	0.5mg
Biotin	5µg
Tris buffer (Sigma Co.)	500mg

Adjust to pH 7.8, dispense in tubes (20ml/tube) and autoclave. Store at 10°C.

Fe-solution

Dissolve 351 mg of $Fe(NH_4)_2(SO_4)_2$. $6H_2O$ and 300 mg of Na_2EDTA in 500 ml of glass-distilled water.

PII metal solution

To 100 ml of glass-distilled water add :

Na_2EDTA	100.0mg
H_3BO_3	114.0mg
$FeCl_3.6H_2O$	4.9mg
$MnSO_4.H_2O$	16.4 mg
$ZnSO_4.7H_2O$	2.2mg
$CoSO_4.7H_2O$	0.48mg

[3] http://www.bio.utexas.edu/research/utex/media/es-enriched-sw.html

4. Long time culturability

4.1. Genomic evolution

Genetic diversity is a balanced interplay between mutation, isolation and natural selection. Genetic variation can be increased by three different strategies: local sequence change, DNA rearrangement and DNA acquisition. Local sequence change is caused by replication infidelity. Internal and environmental mutagens could likewise cause local sequence change as well as DNA rearrangement. Recombinational reshuffling would additionally lead to DNA rearrangements. DNA acquisition would be accomplished by horizontal gene transfer. If these changes in the genome pass the limitation of genetic diversity (restriction-modification systems, repair processes, natural selection) genetically changed species will be isolated from a pool of randomly occurring evolutionary events (Arber, 2000).

4.2. Factors contributing to the generation of genetic variation

Arber (2000) divides the factors that cause genetic variation in two parts: the genetic factors or evolution genes, and the non-genetic factors. The evolution genes are those that represent enzymatic generators of genetic variations (e.g. transposable genetic elements, site-specific DNA inversion occurring at secondary crossing over sites, hypermutable sequences) and the modulators of the frequency of genetic variation (e.g. DNA repair systems, restriction-modification systems).

Among the non genetic factors are the intrinsic instability of nucleotides, the structural flexibility of biologically active molecules (e.g. short-living structural variations of either the DNA substrate or the enzyme protein of infrequently occurring interaction of site-specific recombination enzymes with secondary recombination sites), the random encounter of interactive components (e.g. donor cells, free DNA, virus particles); and chemical and physical mutagens.

4.3. Effect of gene stability on long time culturing

Studies have shown that phenotypically similar populations evolve but underlying genetic divergence may exist because different adaptive mutations can result in similar phenotypic changes. The similarity or difference in genetic changes among replicate populations during adaptive evolution is a function of both the number of different possible adaptive mutations and the frequency at which they occur (Johnson et al., 1995; Kimura, 1983; Travisiano et al., 1996). They are many sources of genetic variations and thus the likelihood of truly parallel genetic changes occurring would appear to be small, given the uncertainties of mutation and fixation (Nakatsu et al., 1998). Investigation of evolution during long time culturing is difficult because improved fitness can result from genetic exchanges associated with a number of different aspects of bacterial growth, including but not limited to the phases of bacterial growth in batch culture, a particular cell structure, or a particular aspect of nutrient utilization (Nakatsu et al., 1998).

Most evolutionary investigations using 10,000 generation experiments are performed using *Escherichia coli* strains. However, one group investigated genotypic evolution with experimental populations of *Ralstonia* sp. (Nakatsu et al., 1998). In a 1,000 generation experiment they observed both parallel and divergent genotypic evolution. In one part of the evolved clones, they observed duplication within the plasmid

(Nakatsu et al., 1998). In 71 of 72 clones a common 2.4 kb PCR product was lost based on PCR amplification using degenerate primers based on repetitive extragenic palindromic sequences. Hybridisation of the fragment from ancestor to DNA from the evolved populations showed that the loss of the PCR product resulted from deletions (Nakatsu et al., 1998). Deletions were also found in the plasmids, but at much lower frequencies (Nakatsu et al., 1998).

It is clear that the frequency of mutation will be dependent on the environmental conditions (substrate, temperature, radiation) (Massey et al., 1999). Still a lot of research needs to be performed in that direction. Also other areas regarding genotypic evolution need to be clarified: the effect of productivity on diversity; the effect of environmental disturbance on diversity; identification of conditions that promote the evolution of specialists versus generalists; identification of conditions that favour phenotypic versus genotypic plasticity; the relationship between random variation, natural selection and adaptation; the genetic causes of adaptive evolution; the nature of the phenotype-to-genotype map; and the importance of modularity in evolution (Rainey et al., 2000).

5. Development of a MELISSA Genome Watch website

5.1. Introduction

A portal site to keep track of genome data relevant to the MELISSA project was established on the BSCW server[1] and made accessible to all registered partners. This website (**melgen**[2]), which is restricted to enlisted members and protected by a password, basically consists of a table (Fig. 1) containing, or hyperlinking to, genome data of the following organisms:

(i) existing or putative members of the bacterial consortium in the decomposing compartment (C1) (e.g., *Clostridium thermocellum*)
(ii) principle MELISSA organisms *Rhodospirillum rubrum* (C2), *Nitrosomonas europaea* and *Nitrobacter winogradskyi* (C3), and *Arthrospira platensis* (C4)
(iii) other relevant organisms (OROs) which are either phylogenetically related to the organisms listed in (i) and (ii), possibly providing an insight into the physiology and genetics of the C1-C4 MELISSA strains, or which may serve as reference organisms for sensory or analytical purposes.

The structure of the underlying table has been kept to a bare minimum, with only seven informative fields. These fields may be emended or extended according to future needs and suggestions of the MELISSA partners. Information will be updated on a regular basis (i.e., once a month) and more organisms may be added in due course (i.e., C1 organisms, OROs, etc.).

5.2. Strain information

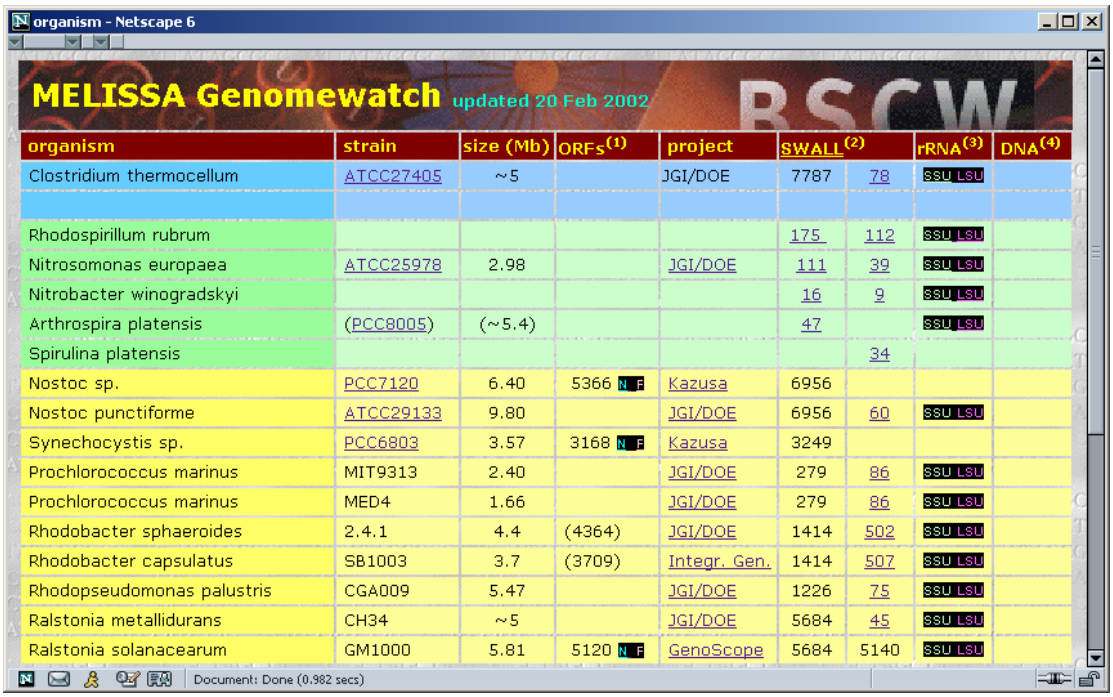
Obviously, a distinction must be made between the individual strains of various genome projects (as is the case for *Prochlorococcus marinus*), and for each strain

[1] BSCW (Basic Support for Cooperative Work) for collaboration over the Web (http://bscw.gmd.de/)
[2] http://bscw.gmd.de/bscw/bscw.cgi/d33092023-1/*/index.html

separate data will be included in the **melgen** table. However, as we will see further (field 5), sequence data linked from within the table do not necessarily correspond with a given strain but rather connect to the genus or species to which this strain belongs. Where possible, the strain notation is linked to the culture collection holding that strain.

5.3. Genome size

The approximate size of the genome is given in milion bases (Megabase, Mb). For completed or nearly completed sequencing projects an accurate estimate is possible, but for ongoing genome projects or unsequenced genomes, only a provisory size can be given. The size of the genome allows a rough estimate of the expected number of open reading frames (ORFs) because, for most prokaryotes, the average size of an ORF is ca. 0.9 kilobase (kb).



organism	strain	size (Mb)	ORFs(1)	project	SWALL(2)		rRNA(3)	DNA(4)
Clostridium thermocellum	ATCC27405	~5		JGI/DOE	7787	78	SSU LSU	
Rhodospirillum rubrum					175	112	SSU LSU	
Nitrosomonas europaea	ATCC25978	2.98		JGI/DOE	111	39	SSU LSU	
Nitrobacter winogradskyi					16	9	SSU LSU	
Arthrospira platensis	(PCC8005)	(~5.4)			47		SSU LSU	
Spirulina platensis						34		
Nostoc sp.	PCC7120	6.40	5366 N F	Kazusa	6956			
Nostoc punctiforme	ATCC29133	9.80		JGI/DOE	6956	60	SSU LSU	
Synechocystis sp.	PCC6803	3.57	3168 N F	Kazusa	3249			
Prochlorococcus marinus	MIT9313	2.40		JGI/DOE	279	86	SSU LSU	
Prochlorococcus marinus	MED4	1.66		JGI/DOE	279	86	SSU LSU	
Rhodobacter sphaeroides	2.4.1	4.4	(4364)	JGI/DOE	1414	502	SSU LSU	
Rhodobacter capsulatus	SB1003	3.7	(3709)	Integr. Gen.	1414	507	SSU LSU	
Rhodopseudomonas palustris	CGA009	5.47		JGI/DOE	1226	75	SSU LSU	
Ralstonia metallidurans	CH34	~5		JGI/DOE	5684	45	SSU LSU	
Ralstonia solanacearum	GM1000	5.81	5120 N F	GenoScope	5684	5140	SSU LSU	

*Fig. 1: layout of the **melgen** portal site*

5.4. Open reading frames (ORFs)

The exact number of ORFs is given for published genomes only, while accurate estimates (in parantheses) are given for projects in progress. It is important to realise that the actual number of ORFs depends on which gene identification algorithm was used and on the accuracy of the DNA sequence (e.g., authentic frameshifts versus sequencing errors). For each published genome, two hyperlinks are provided. The first one is to the corresponding NCBI genome site, the other one links directly – were applicable – to the original FTP site of the genome project.

5.5. Genome Project

This field basically is a hyperlink to the original genome project's website. If there are multiple genome websites available for the same project (i.e. in collaborative sequencing efforts, several partners may have an individual webpage), the most informative website is choosen.

5.6. Protein sequence data

Protein sequences are retrieved from the SWALL[3] database of protein sequences via the extended search application of the Sequence Retrieval System (SRS)[4] using either the genus name (1st column) or species name (2nd column) as keyword. For each search, a count is given. Comparison of the counts gives some insight in how much genomic information is available for each species versus genus. For instance,78 protein sequences were found for the species *Clostridium thermocellum*, while for the genus *Clostridium* 7787 entries were found. This is due to the fact that the genomes of *Clostridium acetobutylicum* and *Clostridium perfringens* are published. Thus, although genome data for *C. thermocellum* appears to be very limited, a massive amount of genome data is available for other clostridial species. Without such counts, this kind of information would remain invisible. Hyperlinks to SWALL entries are provided at the species level (not for published genomes) or at the genus level for MELISSA strains for which no project data are available. Webpages containing SWALL data were generated by SRS and were customized manually. All SRS-generated pages contain further links to Swiss-Prot and/or Genbank information (Fig.2).

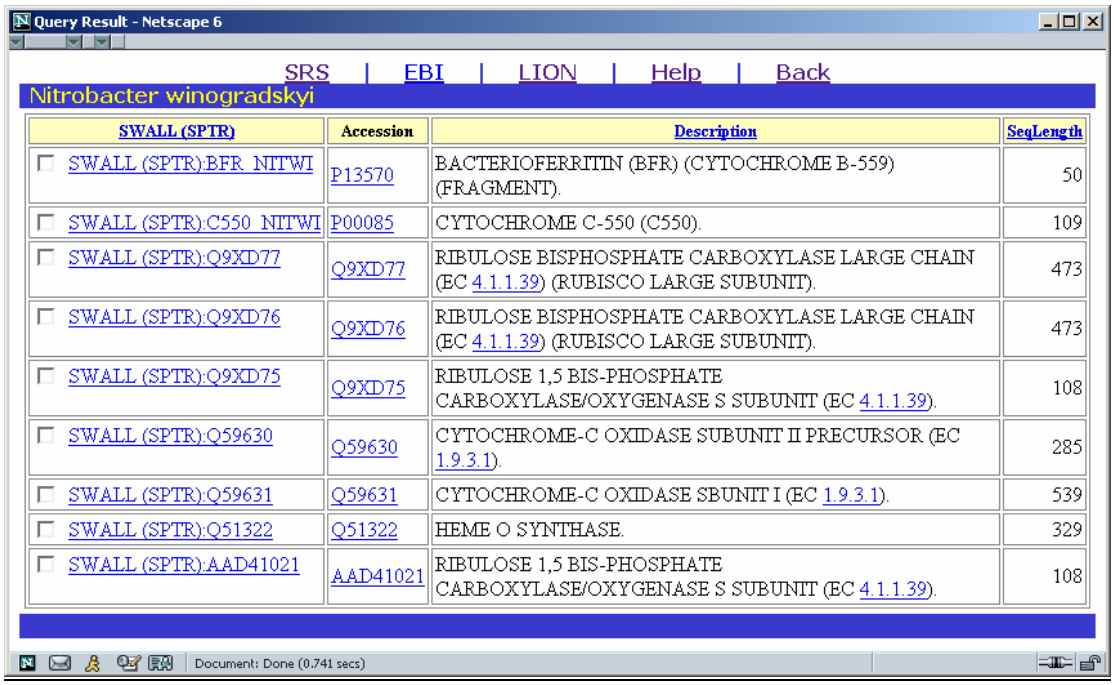


Fig. 2: SRS-generated pages after customization

[3] non-redundant protein database consisting of SWISS-PROT, SpTrEMBL, and TrEMBLnew
[4] http://srs6.ebi.ac.uk/

5.7. Ribosomal RNA sequence data

Using the EMBL[5] database as source, 16S and 23S rRNA gene sequences were retrieved via SRS for each given **species**, where applicable and available. Webpages generated by SRS were manually edited. All SRS-generated pages contain further links to Swiss-Prot and/or Genbank information (similar to Fig.2)

5.8. DNA data

The last field has been reserved to include DNA data. It is not the intention to provide whole genome data here (such data can be find via field 4 "Genome Project"), rather specific data in accordance to the needs of the partners. This could be mobile elements (IS, transposon, etc.), repetitive elements (REP, ERIC, etc.), particular intergenic regions (intergenic spacers [e.g. PC-IGS, gvpA-IGS], internal transcribed spacers [e.g. rDNA-ITS]), regulatory elements, or any other characteristic DNA feature with relevancy to the project.

6. Critical points

6.1. Description of strains and important genes

The proteomic approach allows to detect and identify specific surface and excreted proteins, which can be used for description of the MELiSSA strains. On the other hand is it possible to identify the strains by 16 or 23S ribosomal DNA sequences at the genetic level. Critical genes however will be far more complicated to discover. A first approach will concentrate on the detection of the genes involved in DNA repair, stress, the functions that are involved in more macroscopic genetic rearrangements, and important product processing genes using the sequenced genomes of closely related organisms using PCR-based membrane/chips technology. Likewise, on the basis of known gene sequences of these sequenced reference strains, the nucleotide sequence of the corresponding genes of the MELiSSA strains can be extracted by the use of degenerate oligonucleotides and subsequent amplification can provide the sequence of the targeted parts of the gene of interest. After having identified the most important genes, these will be sequenced and used for monitoring gene stability in the MELiSSA strains. An important contribution from genomic data (i.e. catalogue of sequenced genes that are available for the MELiSSA strains (*Nitrosomonas*) and bacteria closely related (*R. rubrum* ATCC 11170) or not so closely related (*Synechocystis*) to the MELiSSA strains, has been made available and will be updated regularly.

6.2. Monitoring stress

In a first step the effects of stresses like oxidative stress, temperature, supernatant and nutrient starvation will be investigated at the genetic and proteomic level. In a later stage the effect of space related stresses like proton- and α-radiation or microgravity will be investigated, if proper facilities have been made available. Differential expression, caused by various stresses, can be investigated at the protein level by comparison of the

[5] http://www.ebi.ac.uk/embl/

proteome, extracted from total bacterial pellet or secreted proteins, originating from bacteria having been or not having been exposed to stress. Proteome analysis is in this case a particularly powerful tool for study stress response of different bacterial species. Genomic studies will monitor the induction of stress related genes as well as the mutagenic effect of stress on stress related/DNA repair/product processing genes. In the latter case DNA micro arrays of certain target genes can be used to detect gene expression of the MELiSSA strains under various conditions.

6.3. Monitoring axenicity

Proper functioning of the MELiSSA loop will be depended on several parameters among which the axenic condition is strictly necessary. Even the slightest contamination should be avoided at all times. Therefore it will be of pivotal importance to detect contamination as soon as possible. A spiking experiment will evaluate the efficiency of detecting contaminants by different approaches. The proteomic approach could be used to directly detect the presence of other strains or the effect of the contaminant on the indigenous strain. On the other hand, the proteomic approach can be ideal to identify a typical surface protein for each MELiSSA bacterial culture for further use in flow cytometry. Flow cytometry can detect, count and estimate certain parameters of specific populations of bacteria tagged by using immunolabelling. Next to immunolabelling bacteria can also be visualised the use of fluorescent dyes or autofluorescence as a selective marker of the cells. At the genomic level, PCR based methods will be primarily used to detect contaminants.

Presence of viruses can be detected by extraction of bacteriophage encapsulated DNA, whereafter PCR of 16SrDNA, integrated into phage, can identify the infected host. On the other hand it can be possible to detect infected cells directly by flow cytometry.

6.4. Monitoring genetic stability

Genetic stability of the MELiSSA strains may be affected by mutagenesis or by horizontal gene transfer. Space and processing conditions (oxidizing stress, temperature, nutrient starvation, supernatant, cosmic radiation, microgravity, long time culturing) could act as stressors and if so it is important to investigate to which extent this would effect the genetic integrity of the MELiSSA strains. A reference strain with a completely sequenced genome (*Ralstonia metallidurans* CH34) will be used as a model system to investigate genetic plasticity of bacteria in the presence of stress or during long time culturing. Next to the investigation of mutagenesis, horizontal gene transfer needs to be investigated. Also in this case the reference strain *Ralstonia metallidurans* CH34 will be very useful as a tool to investigate conjugation from and to MELiSSA strains.

Furthermore, it will be very important to investigate the genetic effects on longterm continuous cultivation of every MELiSSA strain. Next to environmental stress, the effect of the supernatans and prolonged culturing it will also be important to investigate the risk of self poisoning through accumulation of intermediary mutagenic or carcinogenic metabolites.

It will be impossible to ensure complete genetic stability during prolonged cultivation of axenic strains. Emphasis should be put on complete relevant phenotypic stability: intact production processing/DNA repair/stress related genes/main metabolic genes, unproblematic growth and biomass production, absence of antibiotic resistence/virulence genes and minimizing mutation stimulating conditions.

ANNEX 1: Literature list: the strains inside the first compartment

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ANNEX 2: Literature list: the photosynthetic bacteria *Rhodospirillum rubrum* and *Arthrospira platensis*

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ANNEX 3: Literature list: the nitrifying bacteria *Nitrosomonas* and *Nitrobacter*

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ANNEX 7: List of sequenced genes of *R. rubrum*.

(Most of the sequenced genes are linked to photosynthesis and to metabolism. Only very few genes seem to be relevant for response to stress. Relevant genes for the MELiSSA project are set in bold. Updated June 2002.)

ppsR gene, *bchG* gene, ORF1, *bchP* gene, ORF2 and ORF3 (partial)
gi|6519335|emb|AJ310779.1 |RRU310779[16519335]

partial *gapl* gene for NAD-dependent glyceraldehyde-3ophate dehydrogenase (phosphorylating)
gi|17976730|emb|AJ252109.1|RRU252109[17976730]

Rhodospirillum rubrum clone pTLI dinitrogenase 3 alpha subunit (*anfD*) gene, partial cds; dinitrogenase 3 delta subunit (*anfG*) gene, complete cds; and dinitrogenase 3 beta subunit (*anfK*) gene, partial cds
Gi|3065898|gb|AF058778. 1 |AF058778[3065898]

branched-chain amino acid aminotransferase (*ilvE*) gene, partial cds; and *GInJ* (*gInJ*) and ammonium transporter AmtB1 (*amtB1*) genes, complete cds
gi| 12642773|gb|AF329498.1|AF329498[12642773]

formyltetrahydrofolate deformylase (*purU*) gene, partial cds; GInK (*glnK*) gene, complete cds; and putative ammonium transporter (*amtB2*) gene, partial cds
gi|6760394|gb|AF207908.1 |AF207908[6760394]

partial *pufM* gene and partial ORF gi|13992592|emb|AJ317975. 1 |RRU317975[13992592]

partial 23S rRNA gene, strain DSM 107 gi|9857106|emb|AJ251267.1|RRU251267[9857106]

por39 and *por41* genes for porin 39 and porin 41 gi|9437318|emb|AJ243942.1|RRU243942[9437318]

pap gene, strain FRl gi|9408181|emb|AJ243818.1|RRU243818[9408181]

atp operon gi|46360|emb|X02499.1|RRATP[46360]

phaCRr gene for PHA synthase gi|6689134|emb|AJ245888. I |RRU245888[6689134]

polyhydroxyalkanoate synthase (*PHA C*) gene, complete cds gi|5823519|gb|AF178117.1
|AF178117[5823519]

(R)-specific trans-2,3-enoylacyl-CoA hydratase (*phaJ*) gene, complete cds
gi|7330731|gb|AF156879.1|AF156879[7330731]

H+ translocating pyrophosphate synthase (*RrPP*) mRNA, complete cds
gi|3002956|gb|AF044912.1|AF044912[3002956]

photosynthetic gene cluster, partial sequence gi|7157953|gb|AF202319.1|AF202319[7157953]

transcriptional activator NifA (*nifA*) gene, complete cds; and NifB (*nifB*) gene, partial cds
gi|4960039|gb|AF145956.1|AM 45956[4960039]

PH protein (*gInB*) and glutamine synthetase (*gh1A*) genes, complete cds
gi|2599563|gb|AF029703.1|AF029703[2599563]

cytoplasmic pyrophosphatase gene, complete cds gi|6650712|gb|AF115341.1|AF115341[6650712]

ADP-glucose pyrophosphorylase (*glgC*) gene, partial cds gi|3834670|gb|AF097739.1|AF097739[3834670]

ribulose-biphosphate carboxylase gene gi|46404|emb|X00286.1|RRRUB1 [46404]

gene for aldehyde dehydrogenase, complete cds gi|4579691|dbj|AB006976.1|AB006976[4579691]

plasmid pKYI DNA for DNA-invertase, complete cds giJ2346993ldbjID17434.1ID17434[2346993]

hd-ald gene for alcohol dehydrogenase, complete cds gi|4519176|dbj|AB023641.1|AB023641[4519176]

bchA gene gi|46376|emb|X65976.1|RRBCHAPUF[46376]

hd-ald gene for alcohol dehydrogenase, complete cds gi|4519176|dbj|AB023641.1|AB023641[4519176]

bchA gene gi|46376|emb|X65976.1|RRBCHAPUF[46376]

CO-induced hydrogenase operon (*cooM, coot', cooL, cooY., cooU, cooH*) genes, iron sulfur protein (*cooF*) gene, carbon monoxide dehydrogenase (*cooS*) gene, carbon monoxide dehydrogenase accessory proteins (*cooC, cooT, cool*) genes, putative transcriptional activator (*cooA*) gene, nicotinate-nucleotide pyrophosphorylase (*nadC*) gene, complete cds, L-aspartate oxidase (*nadB*) gene, and alkyl hydroperoxide reductase (*ahpC*) gene, partial cds gil|515463|gb|U65510.1|RRU65510[1515463]

nicotinamide nucleotide transhydrogenase subunits alpha 1 (*nntAl*) alpha 2 (*nntA2*) and beta (*nntB*) genes, complete cds gi|436912|gb|U01158.1|U01158[436912]

Rhodospirillum rubrum (clone pH2.3) *bcha* gene sequence gi|152589|gb|L10192.1IRSPBCHA[152589]

genes for succinate dehydrogenase cytochrome b small subunit and flavoprotein subunit, complete cds gi|3273339|dbj|AB015756.1|AB015756[3273339]

chlorin reductase subunit (*bchX*) gene, partial cds; and chlorin reductase subunits (*bchY*) and (*bchZ*) genes, complete cds gi|2338766|gb|AF018954.1|AF018954[2338766]

***R.rubrum* 23S rRNA gene gi|2244674|emb|X87290.1|RR23S170S[2244674]**

***Rhodospirillum rubrum* gene for 16S ribosomal RNA gi|494951|dbj|D30778.1|RSP16SRNAK[494951]**

Rhodospirillum rubrum plasmid pKYI pssM gene, complete cds gi|216729|dbj|D12652.1|RSPPKYl [216729]

HMG-CoA lyase (*hmgL*) gene, complete cds gi|1762116|gb|U41280.1|RRU41280[1762116]

cbb operon: cbbE=pentose-5-phosphate 3-epimerase...cbbM=ribulose 1,5bisphosphate carboxylase-oxygenase [Rhodospirillum rubrum, Str-2, Genomic, 3 genes, 1954 nt]
gi|404535|gb|S64484.1(S64484[404535]

nifJ gene gi|453435|emb|X77515.1|RRNIFJ[453435]

***gInB* and *gInA* genes gi|664946|emb|X84158.1|RRGLNBA[664946]**

Rhodospirillum rubrum 5s rRNA gi|46403|emb|X02044.1|RRRN5S[46403]

draT and *draG* genes for ADP-ribosyltransferase and ADPribosylglycohydrolase
R. rubrum cytochrome bcl-complex genes (*petA, petB, petC*) gi|46382|emb|X55387.1|RRCYTBC1[46382]

cycA gene for cytochrome c2 gi|46378|emb|X17605.1|RRCYCA[46378]

gene cluster for F(0)-ATP synthase subunits gi|46369|emb|X12757.1|RRATP2[46369]

***R.rubrum* 16S rRNA gene**
gi|1165141|emb|X87278.1|RR16S107R[1165141]

putative histidine phosphokinase/phosphatase (*ntrB*) gene, partial cds and putative DNA-binding protein (ntrC) gene, complete cds gi|927308|gb|U30377.1|RRU30377[927308]

ribulose bisphosphate carboxylase/oxygenase gene, with lacZ promoter gi|209306|gb|M13162.1 |SYNRUBPS[209306]

S 1 proton-translocating nicotinamide nucleotide transhydrogenase subunit PntAA (pntAA), PntAB (*pntAB*), and PntB (*pntB*) genes, complete cds gi|452571|gb|U05294.1|RRU05294[452571]

Rhodospirillum rubrum (ATCC 11170) photoreaction center H subunit (*puh*) mRNA, 5. end gi|556411|gb|J04820.1 IRSPPUH[556411]

R.rubrum (strain S 1) Leu-tRNA-CAA gi|175879|gb|K00337.1|RSPTRLC[175879]

Phe-tRNA gi|175878|gb|K00331.1|RSPTRF[175878]

Rhodospirillum rubrum (ATCC 11170) 16S ribosomal RNA (partial) gi|175874|gb|M55497.1 |RSPRRI6SA[175874]

small subunit ribosomal RNA gi|175872|gb|M32020.1|RSPRGSSA[175872]

ribulose bisphosphate carboxylase, large subunit gene gi|152622|gb|K01999.1|RSPRUBPL[152622]

RNase P RNA (rnpB) gene, complete sequence gi|152621|gb|M59355.1|RSPRNPB[152621]

3-phospho-D-glycerate carboxy-lyase (rbcR) gene, 5' end gi|152619|gb|M21799.1|RSPRBCR[152619]

photoreaction center L and M subunit genes, complete cds gi|1526161gbIJ03731.1 IRSPPUFLM[152616]

puf gene operon *pufB* gene encoding beta polypeptide of the B880 antenna, 5' end gil152615igbIAH000927.1,SEG RSPPUFAO[152615]

puf operon *pufM* gene encoding the M polypeptide, 3' end gi|l52614|gb|M20899.1|RSPPUFA03[152614]

puf operon *pufA* gene encoding alpha polypeptide of the B880 antenna, 3' end, and puff, gene, 5' end gi|152613|gb|M20898.1|RSPPUFA02[152613]

puf gene operon *pufB* gene encoding beta polypeptide of the B880 antenna, Tend gi|152612|gb|M20897.1|RSPPUFA01[152612]

Rhodospirillum rubrum (ATCC 11170) photoreaction center H subunit (*puh*) mRNA, 5. end gi|556411|gb|J04820.1|RSPPUH[556411]

dinitrogenase reductase (ni" gene, complete cds, and dinitrogenase alpha subunit (*nifD*') gene, 5' end gi|152609|gb|M33774.1|RSPNIFHD[152609]

Rhodospirillum rubrum B880 holochrome gene, complete cds gi|l52606|gb|M11801.1|RSPHOC[152606]

ferredoxin I (*fdxN*) gene, complete cds gi|152604|gb|L11914.1|RSPFDX[152604]

ATP synthase (F-0 sector) gene, complete cds gig 1525961gbIM37308. I IRSPFOATP[152596]

R.rubrum (strain S 1) Leu-tRNA-CAA gi|175879|gb|K00337.1|RSPTRLC[175879]

Phe-tRNA gi|175878|gb|K00331.1|RSPTRF[175878]

Rhodospirillum rubrum (ATCC 11170) 16S ribosomal RNA (partial) gi|175874|gb|M55497.1|RSPRRI 6SA[175874]

small subunit ribosomal RNA gi|l75872|gb|M32020.1|RSPRGSSA[175872]

ribulose bisphosphate carboxylase, large subunit gene gi|152622|gb|KO 1999.1|RSPRUBPL[152622]

RNase P RNA (mpB) gene, complete sequence gi|l52621|gb|M59355.1|RSPRNPB[152621]

3-phospho-D-glycerate carboxy-lyase (rbcR) gene, Tend gi|152619|gb|M21799.1|RSPRBCR[152619]

photoreaction center L and M subunit genes, complete cds gi|152616|gb|J03731.1|RSPPUFLM[152616]

puf gene operon *pufB* gene encoding beta polypeptide of the B880 antenna, Tend gi|152615|gb|AH000927.1|SEGRSPPUFAO[152615]

puf operon *pufM* gene encoding the M polypeptide, 3' end gi|152614|gb|M20899.1|RSPPUFA03[152614]

puf operon *pufA* gene encoding alpha polypeptide of the B880 antenna, 3' end, and pufL gene, 5' end gi|152613|gb|M20898.1|RSPPUFA02[152613]

puf gene operon *pufB* gene encoding beta polypeptide of the B880 antenna, Tend gi|152612|gb|M20897.1|RSPPUFA01[152612]

ANNEX 8: List of sequenced genes of *Spirulina platensis*.

(Relevant genes for the MELiSSA project are set in bold. Updated June 2002.)

apcA, apcB and *apcC* genes gi|17974192|emb|X95898.2|SPAPCABGN[17974192]

cyaG gene for adenylate cyclase, complete cds gi|11990886|dbj|D49531.1|D49531[11990886]

DNA for phytoene synthase, complete cds gi|2189954|dbj|AB001284.1|AB001284[2189954]

delta 9 fatty acid desaturase (*desC*) gene, partial cds gi|4100827|gb|AT002252.1|AF002252[4100827]

DNA binding response regulator *RpaB* gene, partial cds gi|4809168|gb|AF135392.1|AF135392[4809168]

cpc operon, complete sequence gi|5726477|gb|AF164139.1|AF164139[5726477]

***rps10* and *tuf* genes gi|406273|emb|Z21676.1|SPRPSl 0A[406273]**

desC gene gi|2576328|emb|AJ002065.1|SPAJ2065[2576328]

DNA for allophycocyanin alpha subunit, allophycocyanin beta subunit, complete cds gi|2116750|dbj|D86179.1|D86179[2116750]

serine esterase [Spirulina platensis, C1, Genomic, 827 nt] gi|546788|gb|S70419.1|S70419[5467881]

cpcB and *cpcA* gene gi|1654092|emb|Y09074.1|SPCPCBAGE[1654092]

desD gene gi|809109|emb|X87094.1|SPDESDGEN[809109]

***str* operon containing the *rpsL* gene, *rpsG* gene, *fus* gene and *tuf* gene for ribosomal protein S 12, ribosomal protein S7, translation elongation factor EF-G and translation elongation factor EF-Tu respectively gil47447lembIX15646.1 ISPSTR[47447]**

rpsB gene for ribosomal protein S2 and *tsf* gene for elongation factor Ts gi|296029|emb|X53651.1|SPRPSTSF[296029]

desA gene gi|805063|emb|X86736.1|SPDESAGE[805063]

gene for ATPase gamma subunit gi|577820|emb|Z46799.1|SPATPSGSU[577820]

(3R)-hydroxymyristoyl acyl carrier protein dehydrase (*fabZ*) homolog gene, partial cds gi|1145807|gb|U41821.1|SPU41821[1145807]

RecA (*recA*) gene, complete cds gi|976444|gb|U33924.1|SPU33924[976444]

Spirulina platensis acetohycroxy acid synthase (ilvY), 5' end gi|152906|gb|M75907.1|SPUAHASL[152906**]**

acetohydroxy acid synthase (*ilvX*), partial cds gi|152904|gb|M75906.1|SPUAHASK[l 52904]

3-isopropylmalate dehydrogenase (*leuB*) gene, Send gi|l52902|gb|M75903.1|SPU31D[152902]