MICROARRAY ANALYSIS OF THE EFFECTS OF HEAT AND COLD STRESS ON HYDROGEN PRODUCTION METABOLISM OF RHODOBACTER CAPSULATUS

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ABSTRACT

MICROARRAY ANALYSIS OF THE EFFECTS OF HEAT AND COLD STRESS ON HYDROGEN PRODUCTION METABOLISM OF RHODOBACTER CAPSULATUS

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Rhodobacter capsulatus DSM1710 is a purple non-sulfur bacterium capable of hydrogen production via photofermentation. Biohydrogen is a clean and renewable way of hydrogen production, which can be achieved by PNS bacteria in outdoor large scale photobioreactors using sun light. In outdoor conditions bacteria can be exposed to heat and cold stress. In this study in order to understand the effects of heat and cold stress on photofermentative hydrogen production and gene expression profile of *R.capsulatus* on acetate as the carbon source, microarray analysis was carried out. Since there is no commercially available microarray chip for *R.capsulatus*, an Affymetrix GeneChip® was designed and it was manufactured by Affymetrix.

The experiments were conducted at 30 °C as the control condition, 42 °C for heat stress and 4 °C for cold stress under constant illumination of 2000 lux. Growth of bacteria, pH of the media, hydrogen production and acetate consumption were

determined periodically. Upon heat and cold stress hydrogen production was stopped, while bacterial growth continued under cold stress but stopped under heat

stress. Acetate could be utilized totally under cold stress, however, some acetate remained in the medium after heat stress application. Microarray analysis revealed that genes taking role in nitrogen metabolism, photosynthesis and electron transport, which are core metabolisms for hydrogen production were highly up regulated under cold stress, while down regulated under heat stress, meaning that heat stress inhibited hydrogen and energy production of *R.capsulatus*. Moreover, cell envelope and related transporter and binding proteins were negatively affected by heat stress.

Keywords: *Rhodobacter capsulatus*, biohydrogen production, microarray, heat stress, cold stress

ÖZ

SICAK VE SOĞUK STRESİNİN RHODOBACTER CAPSULATUS BAKTERİSİNİN HİDROJEN ÜRETİM METABOLİZMASI ÜZERİNE ETKİLERİNİN MİKRODİZİN YÖNTEMİ İLE İNCELENMESİ

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Rhodobacter capsulatus DSM1710 mor, kükürtsüz ve fotofermentasyon yoluyla hidrojen üretebilen bir bakteridir. Biyohidrojen üretimi, mor kükürtsüz bakterilerle dış ortam koşullarında büyük ölçekte, güneş enerjisi kullanarak yapılabildiğinden hidrojen üretiminin temiz ve yenilenebilir bir yoludur. Dış ortam koşullarında bakteriler sıcak ve soğuk stresine maruz kalabilirler. Bu çalışmada, sıcak ve soğuk stresinin *R.capsulatus* bakterisinde asetat kullanılarak yapılan fotofermentatif hidrojen üretimi ve gen ifadelenme profili üzerindeki etkilerinin anlaşılması amacıyla mikrodizin analizi yapılmıştır. *R.capsulatus* için hazır bir mikrodizin çipi bulunmadığından, bu bakteri için bir çip tasarlanmış ve Affymetrix firmasına ürettirilmiştir. Deneyler 30 °C kontrol, 42 °C sıcak stresi ve 4 °C soğuk stresi olacak şekilde 2000 lux sabit ışık yoğunluğu altında yapılmıştır. Bakteri büyümesi, ortam pH'sı, hidrojen üretimi ve asetat kullanımı periyodik olarak ölçülmüştür. Sıcak ve soğuk stresi hidrojen üretimini durdurmuştur. Soğuk stresi altında bakteri büyümesi devam ederken, sıcak stresi altında bakteri büyümesi de durmuştur. Soğuk stresi altında asetatın tamamı tüketildiği halde sıcak stresi altında ortamda bir miktar asetat

kalmıştır. Mikrodizin analizleri, hidrojen üretimi için gerekli olan azot metabolizması, fotosentez ve elektron transfer metabolizmaları genlerinin ifadelerinin soğuk stresi altında arttığını, sıcak stresi altında ise azaldığını göstermiştir. Bu sonuçlar sıcak stresinin hidrojen üretim metabolizmasını engellediğini göstermektedir. Ayrıca hücre zar ve duvarı ile ilgili metabolizmalar ile taşıyıcı proteinler sıcaklık stresinden olumsuz etkilenmiştir

Anahtar kelimeler: *Rhodobacter capsulatus*, biyohidrojen üretimi, mikrodizin, sıcak stresi, soğuk stresi

To Mamiçka

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LIST OF SYMBOLS AND ABBREVIATIONS

A	Irradiated area (m^2)
Acetyl-CoA	Acetyl coenzyme A
ADP	Adenosine di-phosphate
ANOVA	Analysis of variance
ATP	Adenosine tri-phosphate
B. subtilis	*Bacillus subtilis*
cDNA	Complementary DNA
DFE	Dark fermenter effluent
DNase	Deoxyribonuclease
E.coli	*Escherichia coli*
Fd	Ferrodoxin
Fe	Iron
GC	Gas chromatography
GCOS	GeneChip® Operating Software
gdw	Gram dry weight
HPLC	High performance liquid chromatography
hup^-	Uptake hydrogenase deficient
I	Light intensity (Watt/m^2)
mmol	Millimole
Mo	Molybdenum
NAD	Nicotinamide adenine dinucleotide
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction

PNS	Purple non-sulfur
R	Universal gas constant
R. capsulatus	*Rhodobacter capsulatus*
RNA	Ribonucleic acid
R. palustris	*Rhodopseudomonas palustris*
rRNA	Ribosomal RNA
R.rubrum	*Rhodospirillum rubrum*
R. sphaeroides	*Rhodobacter sphaeroides*
t	Time (hour)
TCA	Tricarboxylic acid
TE	Tris EDTA
tRNA	Transfer RNA
UV	Ultraviolet
V	Volume (ml or L)

CHAPTER 1

INTRODUCTION

1.1 Hydrogen as an Energy Carrier

The world's energy demand is increasing. The energy consumption rate is predicted to increase by 44% by the year 2030 (McKinlay and Harwood, 2010). Today, the energy demand of the world is mostly (80%) met by fossil fuels. Fossil energy resources are finite and this level of consumption will eventually lead their depletion. The combustion of fossil fuels emits excessive amounts of CO_2 and organic compounds like sulfur oxides (SO_x) and nitrogen oxides (NO_x), thus polluting the atmosphere. Rapid urbanization and industrialization contribute highly to the increase in energy demand and pollution (Das and Veziroglu, 2001). Atmosphere pollution causes global changes such as global warming which threaten the life on Earth. In order to replace fossil fuels, a renewable and environmentally safe energy source is needed and hydrogen is suggested as an alternative to fossil fuels. Hydrogen is the lightest and most abundant element in the universe. Among all the fuels, hydrogen has the highest gravimetric energy density (Levin et al, 2004). Combustion of hydrogen yields water as the main product, thus it is regarded as a clean and non-polluting fuel. It is expected that hydrogen will be used in fuel-cell powered vehicles in the near future. Hydrogen-powered vehicles have been developed but are still under optimization by car-manufacturing companies (Akkerman et al., 2002, Ho and Karri, 2010).

1.2 Hydrogen Production

Hydrogen can be produced from gaseous or liquid fuels by various processes. As a secondary form of energy, it is produced by using fossil fuels (coal, petroleum, natural gas), nuclear reactors (fission reactors and breeders) and renewable energy sources (hydroelectric power, wind power, solar thermal systems, etc.). The primary fuel used and the chemical reactions involved determine the process of hydrogen production. It is seen from Figure 1.1 that only 4 % of hydrogen is produced by a process other than the direct usage of fossil fuels. Of the fossil fuels, steam reforming of natural gas has the highest proportion of hydrogen production (Kothari et al., 2008).

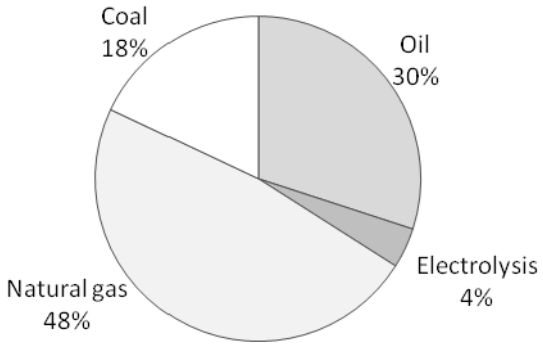


Figure 1. 1 Feedstock used in the current global production of hydrogen (Revised from Kothari et. al, 2008)

Besides steam reforming of natural gas, other methods of producing hydrogen efficiently are electrolysis of water, partial oxidation of hydrocarbons and coal gasification. There are also processes which are under research and development like photochemical and photobiological processes. The Kyoto protocol indicates the reduction of greenhouse gas emissions, since the climatic changes are possible to occur in response to the usage of mainly fossil fuels as the energy source. Thus,

hydrogen production methods without CO_2 emissions are needed. Biological hydrogen production is a promising way of hydrogen production in this sense.

1.3 Biohydrogen

Biological hydrogen production is the production of hydrogen by using microorganisms and a variety of renewable resources (Levin et al., 2004).

Microorganisms generate hydrogen to either dispose of excess reducing equivalents, or to fix nitrogen through which hydrogen is produced as a byproduct (Kotay and Das, 2008). A lot of microorganisms are capable of producing hydrogen since they have hydrogen producing enzymes. These enzymes, which are known to have complex metallo-clusters as active sites, catalyze the simple reaction: (Hallenbeck and Benemann, 2002)

$$2H^+ + 2e^- \leftrightarrow H_2 \tag{1.1}$$

Das and Veziroğlu (2001) classified the biohydrogen production processes as follows:

- Biophotolysis of water by algae and cyanobacteria
- Fermentative hydrogen production from organic compounds
- Photodecomposition of organic compounds by photosynthetic bacteria
- Hybrid systems using fermentative and photosynthetic bacteria

Biological hydrogen production processes can be divided into two groups: light-dependent and light-independent process. Biophotolysis and photofermentation are light-dependent, whereas dark fermentation is light independent (Kotay and Das, 2008).

1.3.1 Biophotolysis

Biophotolysis is the decomposition of water into hydrogen and oxygen by microalgae and cyanobacteria using light energy. Biophotolysis is divided into two types: direct biophotolysis and indirect biophotolysis.

Direct photolysis is the process in which light is captured by a photosynthethic apparatus and the recovered energy provides splitting of water into O_2 and H^+. The protons are then reduced to H_2 by the transfer of electrons by ferrodoxin to hydrogenase or nitrogenase enzyme.

The net reaction and the scheme (Figure 1.2) of direct biophotolysis are as follows:

$$4H_2O + \text{“light energy”} \rightarrow 2O_2 + 4H_2 \qquad (1.2)$$

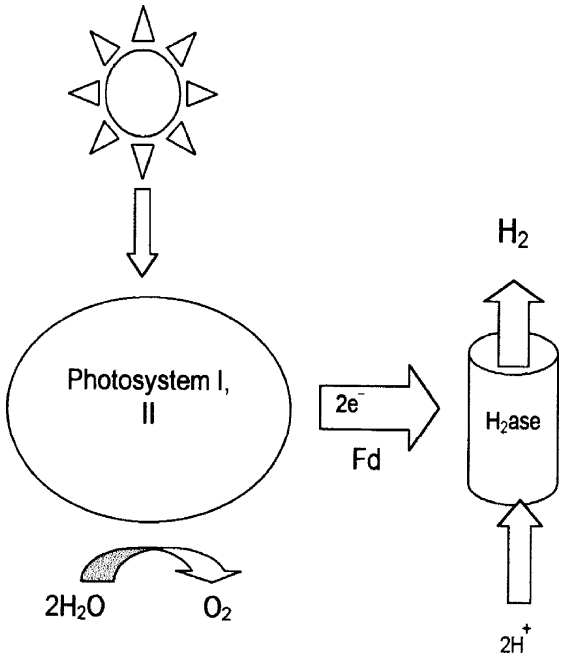


Figure 1.2 Direct biophotolysis (Hallenbeck and Benmann, 2002)

Through this process H_2 can be produced directly from water and sunlight by green algae, but high intensity of light is required and the accumulation of O_2 is inhibitory to the hydrogenase and nitrogenase enzyme system (Nath and Das, 2004). Moreover,

the separation of H_2 and O_2 makes this process economically challenging (Melis, 2002).

Indirect biophotolysis is carried out by cyanobacteria, which are gram positive bacteria that have the same type of photosynthesis as higher plants. They are also nitrogen fixing bacteria possessing hydrogenase and nitrogenase enzyme systems, and they can split water into O_2 and H_2 (Das and Veziroglu, 2001).

The reaction and the scheme (Figure 1.3) of indirect biophotolysis are as follows:

$$12H_2O + 6CO_2 + \text{“light energy”} \rightarrow C_6H_{12}O_6 + 6O_2 \quad (1.4)$$

$$C_6H_{12}O_6 + 12H_2O + \text{“light energy”} \rightarrow 12H_2 + 6CO_2 \quad (1.5)$$

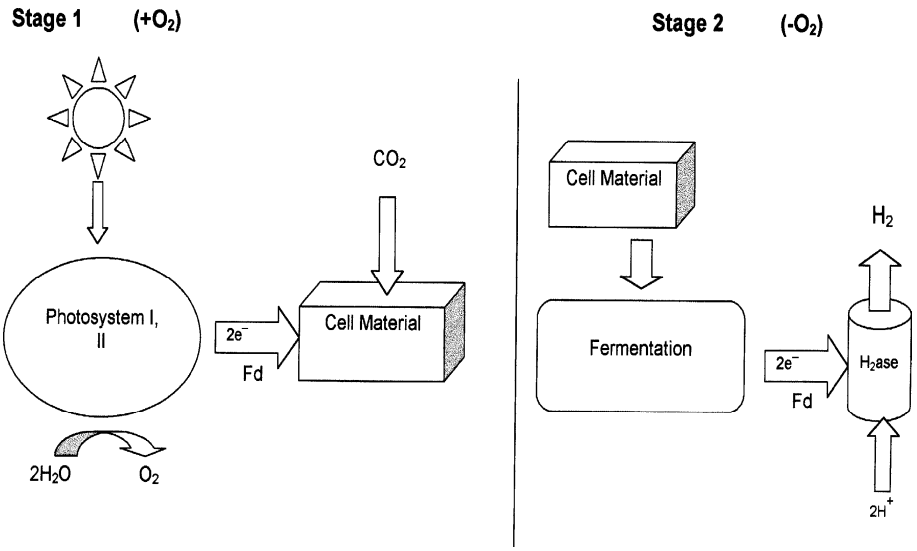


Figure 1. 3 Indirect biophotolysis (Hallenbeck and Benemann, 2002)

O_2 inhibition of the hydrogenase and nitrogenase enzyme systems is avoided in indirect biophotolysis, since CO_2 is first fixed into carbohydrates which are then used to generate H_2. Still, the uptake hydrogenase possesses a problem because it consumes part of the produced hydrogen. Therefore removal of hydrogenase is suggested in order to inhibit hydrogen degredation, thereby optimizing hydrogen yield (Nath and Das, 2004).

1.3.2 Fermentative Production (Dark Fermentation)

Fermentation is an anaerobic process, meaning that there is no oxygen as the electron acceptor in the environment. When bacteria grow heterotrophically on organic substrates, these substrates are degraded by oxidation reactions in order to produce metabolic energy. These oxidation reactions generate electrons which are needed to be disposed of in order to maintain electron neutrality of the cell. In this case, protons act as electron acceptors and are reduced to molecular hydrogen. As shown in Figure 1.4, glucose is initially converted to pyruvate by glycolytic pathways, and then acetyl-CoA is produced upon oxidation of pyruvate. This step requires ferrodoxin reduction. Hydrogenase oxidizes the reduced ferrodoxin and releases electrons to produce molecular hydrogen (Das and Veziroglu, 2008).

Fermentation reactions can be operated at mesophilic, thermophilic or hyperthermophilic temperatures. Usually a mixed biogas containing H_2 and CO_2 is produced, but lesser amounts of CO, CH_4 or H_2S can be produced, too. *Enterobacter*, *Bacillus* and *Clostridium* species are known to produce hydrogen through dark fermentation. The preferred substrates for hydrogen production by this way are carbohydrates like glucose, isomers of hexoses, or polymers like starch. The hydrogen yield differs depending on the fermentation pathway and end product. Theoretically, the maximum hydrogen yield is obtained when the end product is acetate (4 mole H_2 per mole of glucose). Lower hydrogen yield is obtained when the end product is propionate, or with reduced end products like alcohols and lactic acid which contain hydrogen that is not liberated as gas. That is why bacteria should be directed away from alcohols and lactate, but towards volatile fatty acids (Levin et al., 2004).

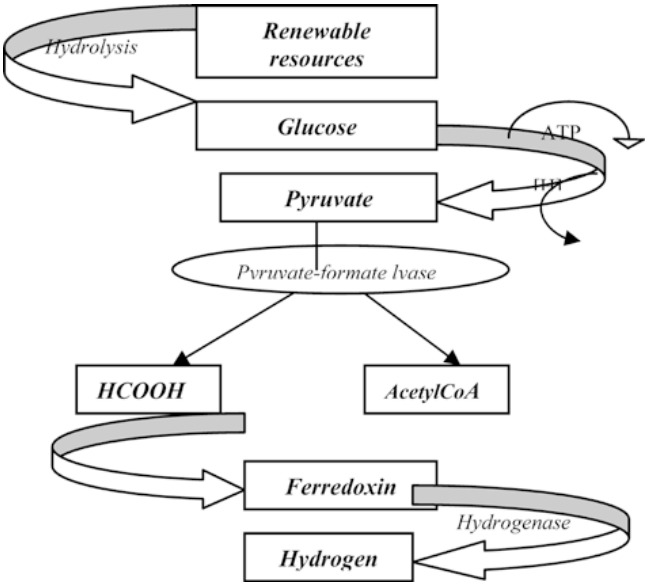


Figure 1. 4 Conversion of renewable resources to hydrogen by fermentation (Nath and Das, 2004)

The major advantages of dark fermentation are that a variety of carbon sources can be used as substrate, valuable metabolites like lactic, butyric and acetic acids are produced, and hydrogen can be produced all day long since it does not require light. Moreover, since it is an anaerobic process O_2 does not limit the process. However, lower yields of hydrogen production and the fact that the reaction becomes thermodynamically unfavorable when hydrogen yield increases are the drawbacks of the process (Nath and Das, 2004).

1.3.3 Photofermentation

Photosynthetic bacteria can produce hydrogen via photofermentation. The capability of using a wide variety of substrates with high substrate conversion efficiencies make photosynthetic bacteria good candidates for hydrogen production. These bacteria have a versatile metabolic capacities and lack photosystem II, thus no O_2 inhibition of hydrogen production is experienced. They can use a variety of cheap compounds containing organic and inorganic electron sources (Das and Veziroglu, 2008). The scheme of pgotofermentation is shown in Figure 1.5.

Photosynthetic bacteria perform anoxygenic photosynthesis and produce hydrogen in the presence of light. The reaction takes place as follows:

$$CH_3COOH + 2H_2O + \text{“light energy”} \rightarrow 4H_2 + 2CO_2 \quad (1.6)$$

Purple sulfur bacteria produce hydrogen during photoautotrophic growth using reduced sulfur compounds like sulfide, thiosulfide or elementary sulfur as the electron donor. An example to purple sulfur bacteria is *Halorhodospira halophila* in which nitrogenase is the enzyme catalyzing hydrogen production (Tsuihiji et al., 2006).

Purple non-sulfur bacteria produce hydrogen from the degradation of organic substrates such as volatile fatty acids under anaerobic atmosphere using light energy. The culture medium should be nitrogen limited, which means a high C/N ratio. In this environment, bacteria eliminate excess energy through hydrogen production (Koku et al., 2002). The enzyme responsible for hydrogen production is nitrogenase (Figure 1.5). Hydrogen is an obligate product of the nitrogenase reaction, in which nitrogenase acts as an ATP-powered hydrogenase in the absence of N_2. (McKinlay and Harwood, 2010).

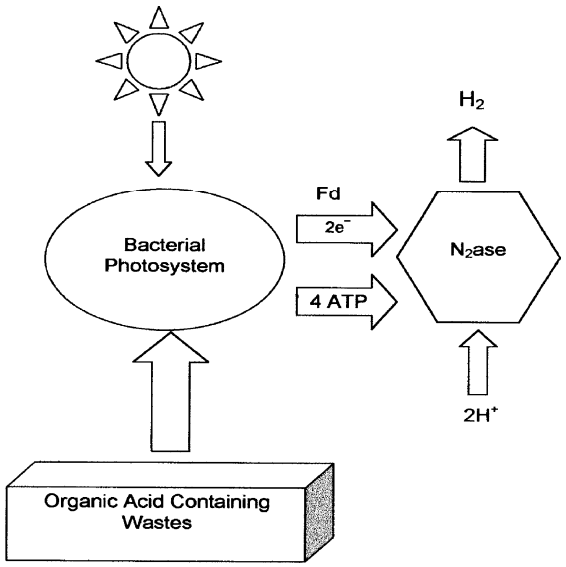


Figure 1. 5 Photofermentation (Hallenbeck and Benemann, 2002)

A large spectrum of light and different waste materials such as distiller effluents can be used by photosynthethic bacteria. However, the light conversion efficiency in this process is low, only 1-5%.

1.3.4 Integrated Systems

The end products of dark fermentation are short chain organic acids (acetate, lactate, and butyrate) and alcohols. Photofermentative bacteria can utilize the end products of dark fermentation. Dark fermentation followed sequentially by photofermentation is called an "integrated system" for hydrogen production. The integrated process maximizes the overall H_2 yield and conversion efficiency of a substrate (Nanqi et al, 2011).

In dark fermentation 4 moles of H_2 is theoretically produced from glucose if the end product is acetate. Additional 8 moles of H_2 can be theoretically produced by photofermentation using the products of dark fermentation. Therefore, with an integrated system 12 moles of hydrogen can be produced from 1 mole of glucose, as seen from the following reactions (Kotay and Das, 2008).

Dark fermentation;

$$C_6H_{12}O_6 + 2H_2O \rightarrow 4\ H_2 + 2CH_3COOH + 2\ CO_2 \quad (1.7)$$

Photofermentation;

$$2CH_3COOH + 4H_2O + \text{"light energy"} \rightarrow 8H_2 + 4CO_2 \quad (1.8)$$

Integrated System;

$$C_6H_{12}O_6 + 6\ H_2O \rightarrow 12\ H_2 + 6\ CO_2 \quad (1.9)$$

A good example for the integrated systems is the EU 6th framework integrated project HYVOLUTION, non-thermal production of pure hydrogen from biomass.

The goal of this project was to produce cost effective pure hydrogen using multiple biomass feedstocks in a two stage process (Claassen and Vrije, 2006). In the first stage different feedstocks were converted into hydrogen, CO_2 and organic acids by thermophilic bacteria. In the second stage, organic acids present in the effluents of the dark fermentation were converted to H_2 and CO_2 by photofermentation. By this novel approach of HYVOLUTION highest hydrogen production efficiency was aimed to achive. Thoughout this project, feedstocks like sugar beet molasses (Özgür et al., 2010), potato steam peel (Afşar et al., 2011), miscanthus (Uyar et al., 2009) were used and hydrogen was produced by sequential dark and photofermentation processes. METU Biohydrogen group was a member of HYVOLUTION and the coordinator of workpackage 3 which focuses on photofermentative hydrogen production from organic acids to increase yields and construct large scale photobioreactors for hydrogen production.

1.4 Purple Non-sulfur Bacteria

Purple non-sulfur (PNS) bacteria are anoxygenic phototrophic α-proteobacteria. Proteobacteria are one of the largest phyla among bacteria, comprising 45% of cultured bacteria. They have photosynthetic capability (Gupta, 2010) and can grow phototrophically under anoxygenic conditions without producing oxygen. They use organic substrates as both a carbon source and as photosynthetic electron donors. Proteobacteria have only one reaction center and quinones as electron acceptors (Imhoff, 2006).

PNS bacteria are ovoid to rod shaped and are motile by polar flagella. They multiply by binary fission. Their niche is anoxic parts of waters and sediments that receive enough light for phototrophic growth. Having carotenoids and photosynthetic pigments (bacteriochlorophyll a or b) make the cell suspensions yellowish, beige, brown-red or brown depending on the growth condition (Imhoff et al., 2005). The photosynthetic pigments are located in the cell membrane and internal membrane systems and their production depends on the availability of oxygen. Indeed, α-

proteobacteria have extraordinary metabolic versatility. Table 1.2 shows the modes of growth of PNS bacteria.

Table 1. 1 The modes of growth of PNS bacteria

Mode of Growth	**Carbon Source**	**Energy Source**	**Notes**
Photoheterotrophy	Organic carbon	Light	H_2 production and good growth
Photoautotrophy	CO_2	Light	H_2 is consumed in the absence of organic carbon
Aerobic respiration	Organic carbon	Organic carbon	No H_2 production in the presence of O_2
Anaerobic respiration	Organic carbon	Organic carbon	No H_2 production under low light and anaerobic conditions. A terminal electron acceptor other than O_2 (like nitrate) is needed
Fermentation	Organic carbon	Organic carbon	No H_2 production under anaerobic dark conditions

Photoheterotrophy is the preferred growth mode of PNS bacteria, and it is the only mode that results in H_2 production (Koku et al., 2002). Imhoff (2006) indicates that α-proteobacteria require growth factors and vitamins; especially thiamine, niacin and biotin in the media. Moreover, the pH range of 6-9 and temperature of 25-35 °C are optimum for growth (Sasikala et al., 1991).

Rhodobacter capsulatus is a gram negative PNS bacterium belonging to α-proteobacteria. *R. capsulatus* is rod shaped with a diameter of 0.5-1.2 μm. It produces slime and a capsule (Imhoff, 1995). It can store poly-β-hydroxybutyric acid as the storage material. The microscopic image of *R.capsulatus* is shown in Figure 1.6 and the taxonomy is shown in Table 1.3.

Figure 1. 6 The microscopic image of *R. capsulatus* (Institut für Molekulare Enzymtechnologie http://www.iet.uniduesseldorf.de/Frameseiten/Photobiotechnology&topframenav.htm, Last access date: June 26, 2011)

Rhodobacter capsulatus has been studied for its versatile metabolism, hydrogen production and nitrogen fixation. Furthermore, it can be easily mutated by classical procedures, thus offering good opportunities for biochemical and genetic approaches (Weaver et al., 1975).

Table 1. 2 The taxonomy of *R. capsulatus*

Super Kingdom	Prokaryota
Kingdom	Monera
Sub Kingdom	Eubacteria
Phylum	Gracilicutes
Class	Photosynthetic eubacteria
Order	Rhodospirillates
Family	Rhodospirillaceae
Genus	Rhodobacter
Species	Capsulatus

1.5 Hydrogen Production Metabolism of PNS Bacteria

Rhodobacter capsulatus, like other PNS bacteria, produce hydrogen using light energy under anaerobic conditions by the breakdown of organic acids like acetate, lactate, malate. Nitrogen should be limited in the medium. The photosynthetic membrane system, which contains bacteriochlorophyll and caretenoids, plays a role in hydrogen metabolism together with the TCA cycle and the enzyme systems. PNS bacteria produce hydrogen by the action of anaerobic light-dependent TCA cycle, shown in Figure 1.7. The electron produced from the TCA cycle upon oxidation of organic compounds is transferred to the electron carriers in the membrane. As shown in Figure 1.8, the photosystem harvests light, causing a cyclic electron flow within the membrane. This electron flow allows for the generation of a proton gradient which is utilized by ATPase to generate ATP. The protons and electrons produced

through the TCA cycle and the ATP from ATPase are channeled to nitrogenase enzyme which is primarily responsible for hydrogen production. The electrons are transferred to nitrogenase by electon carriers NAD and ferrodoxin (Fd) (Koku et al., 2002). The electron path is as follows:

Substrate→TCA-cycle→NAD/NADH →(Fd)ox/(Fd)red →Nitrogenase (1.10)

1.5.1 Enzymes in Hydrogen Production

The hydrogen production in *R.capsulatus* takes place by the action of two enzymes: hydrogenasen and nitrogenase.

1.5.1.1 Hydrogenase

Hydrogenases are metalloproteins that catalyze the following reaction:

$$H_2 \leftrightarrow 2H^+ + 2e^- \quad (1.11)$$

In PNS bacteria this enzyme acts as a hydrogen uptake enzyme in the presence of H_2, converting molecular hydrogen into protons and electrons; thus it also acts as an electron donor (Kars and Gündüz, 2010). The membrane-bound uptake hydrogenase of *Rhodobacter capsulatus* is synthesized by the *hup* and *hyp* genes (Colbeau et al., 1993). Since uptake hydrogenase decreases the hydrogen production efficiency, this enzyme was eliminated by insertion of an antibiotic resistance gene into *hup*, or by deletion of *hup*. Öztürk et al. (2006) deleted uptake hydrogenase of *R.capsulatus* by interposon mutagenesis and showed that hydrogen production yield and rate together with substrate efficiency were higher than that of the wild type strain. In another study, an uptake hydrogenase deficient *Rhodobacter spaheroides* was obtained by inactivating the enzyme by site directed mutagenesis. Again, higher hydrogen production (20%) was observed with the mutated strain (Kars et al., 2008).

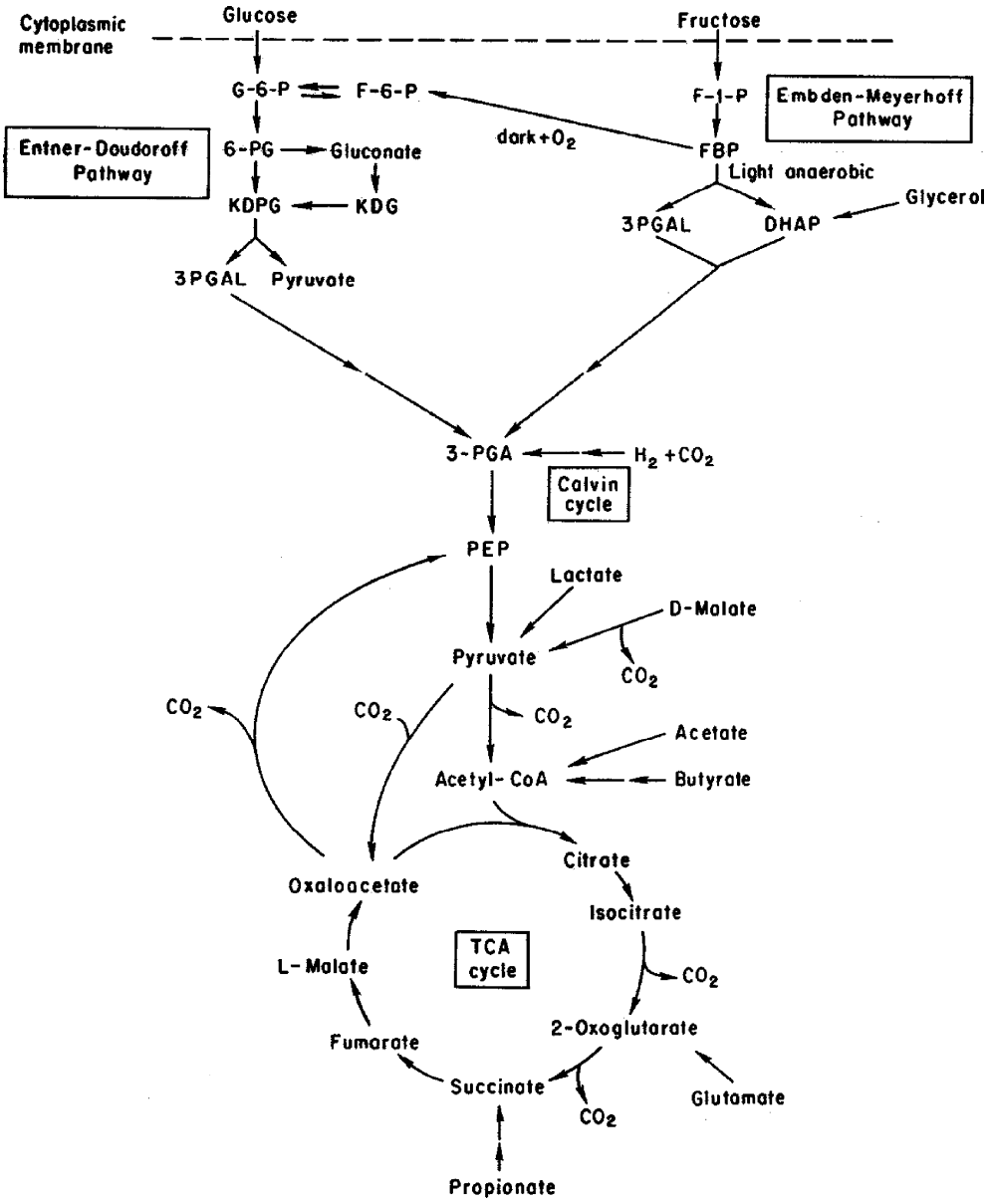


Figure 1. 7 The overall scheme of carbon metabolism in PNS bacteria (Koku et al., 2002)

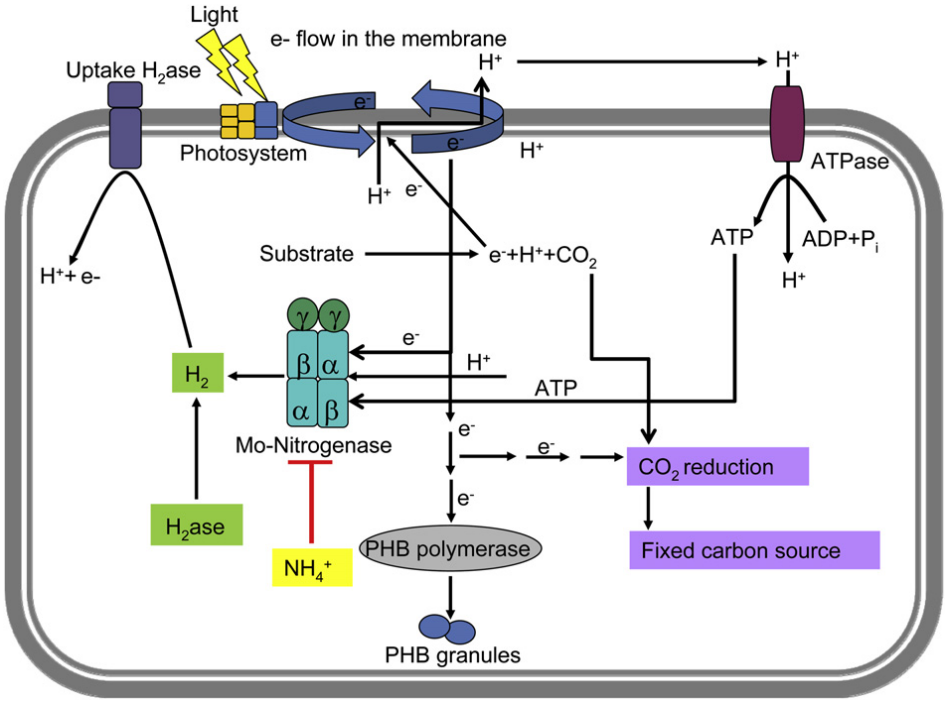


Figure 1. 8 The general scheme of hydrogen production and related metabolism in PNS bacteria (Kars and Gündüz, 2010)

1.5.1.2 Nitrogenase

The role of nitrogenase ezyme is nitrogen fixation; which is defined as the reduction of dinitrogen to ammonia when molecular nitrogen is present by the following reaction:

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i \qquad (1.12)$$

In PNS bacteria, hydrogen production is catalyzed mainl by nitrogenase enzyme is. In the absence of molecular nitrogen and in an anaerobic environment this enzyme catalyzes hydrogen production as seen in the equation:

$$2H^+ + 2e^- + 4ATP \rightarrow H_2 + 4ADP + 4P_i \qquad (1.13)$$

The nitrogenase system contains two component metalloproteins: dinitrogenase reductase (Fe protein) and dinitrogenase (MoFE protein, VFe protein, FeFe protein) (Burris, 1991). *R.capsulatus* has both iron-molybdenum nitrogenase, which is synthesized by *nifHDK* genes, and iron-iron enzyme expressed by *anfHDGK* genes (Haselkorn et al., 2001, Kars and Gündüz, 2010).

Nitrogenase requires electrons and ATP in order to produce hydrogen. The production of hydrogen is an inefficient process in the presence of molecular nitrogen because nitrogen fixation in that condition consumes most of the reducing power. For this reason, to direct nitrogenase for hydrogen production, instead of molecular nitrogen there should be fixed nitrogen sources like glutamate in the environment, and the amount of glutamate should be limited in order to stimulate nitrogenase expression (Kars and Gündüz, 2010). Due to the energy and electron requirement of nitrogenase, its synthesis and activity is under strict control. Oxygen irreversibly destroys nitrogenase because both dinitrogenase and dinitrogenase reductase are sensitive to O_2 (Burris, 1991). PNS bacteria do not produce oxygen during photosynthesis, thus both hydrogenase and nitrogenase are active in anaerobic conditions (Basak and Das, 2007). Another inhibitor of nitrogenase is the presence of ammonium, which represses the synthesis of the enzyme and inhibits its activity. However, this inhibition is reversible and the activity is recovered in the absence of ammonium. Akköse et al. (2009) reported that increasing ammonium concentration decreased and even inhibited hydrogen production over 2mM NH_4Cl. Moreover, the expression of nitrogenase encoding gene *nifH* and its control gene *nifA* were shown to be decreased upon increasing concentrations of ammonium chloride. Nitrogenase synthesis was observed to be light-induced, thus its activity increases by light in *R. capsulatus* (Jouanneau et al., 1985). Having molybdenum in the structure of nitrogenase, PNS bacteria need molybdenum in the medium for hydrogen production. Kars et al. (2006) showed that *nifK* expression in *R. sphaeroides* was reduced when there was no Mo in the medium. Accordingly, in the absence of molybdenum hydrogen was not produced.

1.6 Factors Affecting Hydrogen Production

Hydrogen production by PNS bacteria depend on several factors, like temperature, pH, carbon and nitrogen sources, light intensity, the mode of operation and the employed bacterial strain.

The optimum pH for hydrogen production for *R.sphaeroides* O.U.001 was determined to be in the range of 6-9 by Sasikala et al. (1991). The optimum pH for bacterial growth was found to be at pH 6.5-7.5 (Bergey and Holt, 1994).

The carbon source has an important impact on hydrogen production by PNS bacteria. Barbosa et al. (2001) conducted a study using acetate, malate, lactate and butyrate as carbon sources on some PNS bacteria. The results revealed that acetate is the carbon source used by *Rhodopseudomonas sp*. that gives the highest hydrogen yield. In that study the concentration of acetate was between 6-22 mM, and 22 mM acetate gave the best result. In a study conducted by Özgür et al. (2010) when the initial acetate concentration was 40mM the hydrogen production was highest while the highest substrate conversion efficiency was obtained by 30 mM acetate for *R.capsulatus*. Higher concentrations of acetate were shown to inhibit hydrogen production in a batch immobilized system by Asada et al. (2008). Hillmer and Gest (1977) showed that among the other amino acids glutamate was a good nitrogen source for hydrogen production. They also showed that the concentration of glutamate was important, when they increased the glutamate concentration from 7 to 22 mM there was a progressive decrease in both H_2 formation rate and yield.

Uyar et al. (2007) studied the effect of light intensity on hydrogen production by *R.sphaeroides* O.U. 001. They showed that hydrogen production did not occur in dark. When the light intensity increased hydrogen production increased but it did not increase after 270 W/m^2. The photobioreactors should receive infrared light (750-950 nm) since the bacteriochlorophyll *a* absorbs light at red-infrared region.

The mode of operation influences the amount of hydrogen produced. Avcioğlu et al. (2009) showed that even though the productivity and the yield of hydrogen

production of *R.capsulatus* was lower in continuous operation than in batch, continuous operation could be stable for 3 months, while it was around 150 hours in batch operation. Thus, it was suggested that continuous operation was more suitable for the scale-up and long term hydrogen production.

In order to test efficiencies of different PNS bacterial strains on real DFE of potato steam peel (PSP) having acetate as the carbon source, and an artificial glucose DFE, Afşar et al. (2011) investigated the hydrogen production profiles of *R.capsulatus* and its *hup* deficient strain, *R.sphaeroides* and its *hup* deficient strain and *R.palustris*. They concluded that while *R.sphaeroides* gave the highest hydrogen yield on glucose DFE, *R.capsulatus* gave better results on DFE of PSP. Similarly, in a study conducted by Özgür et al. (2010) on DFE of molasses containing acetate and lactate as carbon source, the *hup*$^-$ mutant of *R.capsulatus* gave better results than *hup*$^-$ strain of *R.capsulatus* and *R.palustris.* Asada et al. (2008) studied hydrogen production by different strains of *Rhodobacter sphaeroides* on acetate and showed that even different strains of the same species have different hydrogen production performances.

Temperature has an important effect on biohydrogen production since the metabolic reactions for hydrogen production occur with the help of enzymes, especially the nitrogenase enzyme. The optimum temperature range for hydrogen production by *Rhodobacter* species is between 31-36 °C (Basak and Das, 2007). Accordingly, Eroğlu et al. (2010) found that the highest hydrogen productivity was at 30 °C by *R.capsulatus*. The expression of *nifA* gene was found to decrease when the temperature was 20 °C and 38 °C compared to 30 °C (Ünlü et al., 2009). Özgür et al. (2010) studied the effect of fluctuating temperatures on hydrogen production of *R.capsulatus hup-* mutant in outdoor photobioreactors of 550 mL volume. They showed a decrease in hydrogen production in daily fluctuations of temperature (15-40 °C) and light/dark cycle. Lack of temperature and light intensity control are the major challenges for the operation of photobioeactors in outdoor conditions. Androga et al. (2011) investigated the factors affecting hydrogen production of *R. capsulatus*

hup$^-$ strain under outdoor conditions in 4 and 8 L photobioreactors and found that during the winter months (October-December) in Ankara the night temperatures were less than 5 °C and hydrogen yield decreased. The total hydrogen productivity in winter was less than during summer (July-August), when the temperature of the photobioreactor was controlled using cooling coils. Avcioğlu et al. (2011) reported that the temperature in the photobioreactor (4 L) could increase above 45 °C in an outdoor operation which decreased hydrogen productivity and yield. They concluded that hydrogen production under natural sunlight was affected greatly by seasonal variations in temperature and suggested that the temperature should not exceed 38 °C in outdoor photobioreactors.

1.7 Effect of Temperature Stress on Bacteria

Any change in the normal environment of the bacteria induces stress on the organisms. Such changes can cause death, growth rate reduction or lag phase increase depending on the extent of the change. Small changes can be tolerated by resisting the stress. The induced stress responses are usually directed at survival rather than growth (Beales, 2004). Among the prokaryotes, the stress responses are very similar. Moreover, some stress response systems like heat shock response are similar in archea and eukaryotes (Ron, 2006).

Outdoor operations are of great importance in terms of scale up for biohydrogen production. The above investigations show that under outdoor conditions bacteria in the photobioreactors experience great temperature changes which can be termed "temperature stress". Changes in environmental factors like temperature, osmolarity or exposure to chemicals can change DNA supercoiling, which affects gene expression of various genes. The supercoiling of DNA acts as a thermosensor, and its regulation is important in terms of DNA related functions like transcription, recombination and replication (Phadtare, 2004).

Previously, some stress studies were conducted on *R.capsulatus* and *R.sphaeroides*. For example, the effects of oxidative stress, 4% ethanol stress, UV radiation and heat shock (42 °C) were studied on protein patterns of *R. sphaeroides* by two dimensional

gel electrophoresis by Nepple and Bachofen (1997) and it was observed modifications in the protein patterns specific to each stress condition. El-Rab et al. (2006) studied how cadmium stress affects the growth, morphology and protein expression in *R.capsulatus* and concluded that the bacteria could grow in 150 μM $CdCl_2$ with induced expression of chaperons. Moreover, changes in membrane lipid composition of *R.sphaeroides* under osmotic stress were studied (Catucci et al., 2004). In this study, the effects of temperature stress on *R.capsulatus* are studied. Temperature is one of the most important factor affecting bacterial growth and development. Temperature stress can be divided into heat and cold stress (or shock) (Panoff et al., 1998).

1.7.1 Heat Shock

Heat shock is a rapid increase in environmental temperature. It causes many physical and chemical changes in bacterial proteins and membranes. Indeed, the heat shock response is stimulated by damaged proteins, the concentrations of which increase in the cell with increase in temperature. Increased temperature induces the expression of heat shock proteins (Hsp) such as proteases and chaperons which reduce the concentration of damaged proteins. Their genes are included in the heat shock stimulon (Ron, 2006). Proteases and chaperons become more important during stress, since the integrity of cellular components affects the viability of the organism. The folding states of proteins are monitored by ATP-dependent proteases and molecular chaperons in a controlled network, and abnormal proteins are degraded by proteases (Gomes and Simão, 2009). Besides gene activation, other methods to cope with heat stress are the initiation of a gene's expression or increase in the rate of transcription of a gene, increasing the life span of mRNA, and stress proteins (Macario and Macario, 2000).

In Gram-negative bacteria the heat shock response is mediated by alternative sigma factors (σ^{32} and σ^{E}) which are transcriptional activators. The sigma factor σ^{32} copes with cytoplasmic protein damage. It regulates the transcription of chaperons like DnaK/DnaJ/GrpE and GroES/EL and the proteases ClpXP, Lon and FtsH.

This sigma factor is encoded by the *rpoH* gene. When the temperature is 30 °C, the cellular concentration of σ^{32} is about 50 molecules per cell. However, when the temperature rises to 42 °C, the amount increases 15-20 fold in 5 minutes, then decreases to 2-3 fold of the 30 °C level (Straus et al., 1987).The other sigma factor σ^{E} is induced upon unfolded outer membrane proteins or periplasmic proteins and thus protects the cell from extracytoplasmic stress. Accordingly, it regulates the transcription of genes for periplasmic folding catalysts, proteases, enzymes taking place in polysaccharide biosynthesis, and cell envelope proteins. The σ^{32} of *E. coli* and that of α-proteobacteria (Ron, 2006) are different; *E.coli* σ^{32} is unstable and controlled through proteolysis by FtsH protease, while in α-proteobacteria it is stable and controlled by a DnaK-mediated mechanism (Nakahigashi et al., 2001).

1.7.2 Cold Shock

Heat shock induces well-defined damage in the cell, like the unfolding of proteins. However, cold shock does not cause well-defined damage. Cold shock affects ribosomes, DNA, and the cell membrane, as shown in Figure 1.9. When the temperature decreases membrane fluidity decreases, protein folding becomes inefficient, ribosome function decreases, and the efficiency of transcription and translation is reduced upon the stabilization of secondary structures of nucleic acids (Phadtare, 2004).

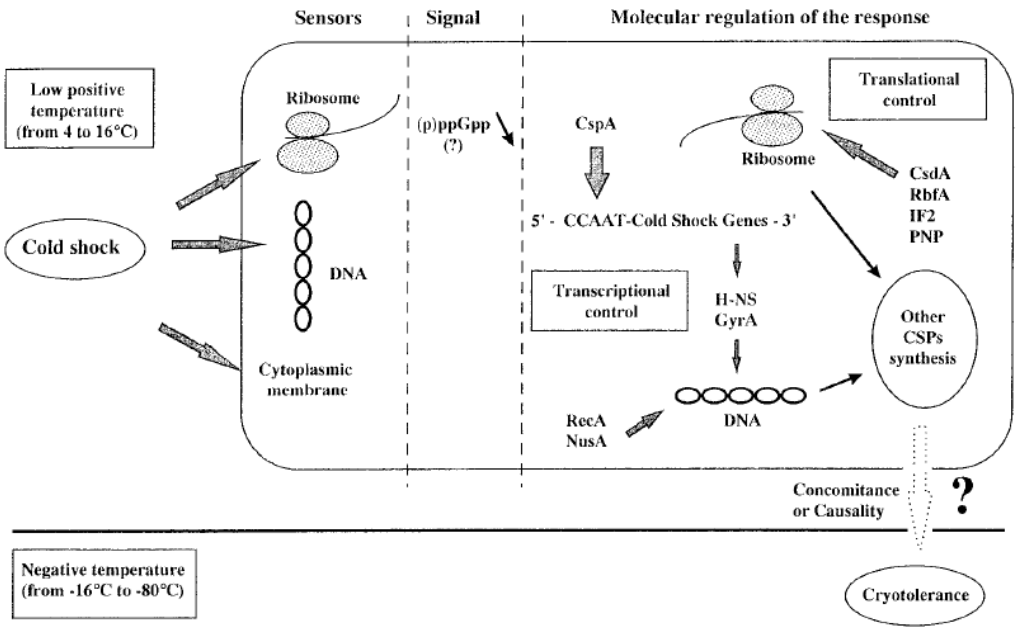


Figure 1. 9 Cold shock responses in *E.coli* and other mesophilic bacteria (Panoff et al., 1998)

Some organisms may produce higher amounts of pigments at below-optimum temperatures than at optimum temperatures. Moreover, the production of flagella is often increased at low temperatures. The production of intracellular or capsular polysaccharides also increases at low temperatures. Fatty acid synthesis and breakdown rates are affected by temperature since the enzymes in fatty acid metabolism are temperature-sensitive. Indeed, membrane lipid composition should be changed for the maintenance of membrane fluidity at lower temperatures (Methe et al., 2005). Changes in cell membrane composition decrease the activity of permeases on the membrane, which in turn reduces solute uptake by the cell. The cell must therefore produce some solutes to control water potential (Ko et al., 1994). Upon temperature decrease, the protein synthesis rate decreases (Farrel and Rose, 1967). Goldstein et al. (1964) showed that at 0 °C the protein synthesis rate was about 350 times slower than 37 °C.

Analogous to the heat shock response, cold shock proteins are induced under low temperatures. In *E.coli* the cold shock proteins are CspA, CspB, CspG, CspI which function as RNA chaperons; CsdA having RNA unwinding activity, RbfA being a binding factor with a role in ribosome maturation. PNP is a ribonuclease cold shock protein. NusA is a cold shock protein, which has a role in the termination and antitermination of transcription. All of these chold shock proteins are grouped as "group I", and they are induced more by cold shock than the "group II" proteins. From the group II proteins, RecA is a recombination factor, IF2 is an initiation factor. Additionally, H-NS is a DNA binding protein, while GyrA is a subunit of topoisomerase DNA gyrase.

1.8 Microarray

The assays of ordered arrangements which are used to study interactions between biological molecules are called "arrays". The miniaturized, small format arrays are called "microarrays". The biological material used in microarrays can be DNA, proteins or carbohydrates, while DNA microarrays are the most frequently used for analysis of gene expression or single nucleotide polymorphism (SNP) screening (Falciani, 2007). In DNA microarrays, a mixture of labeled nucleic acids which are called "target" are hybridized with the nucleic acids called "probes". Probes are located on glass, silicon or plastic surfaces and the location of a probe on the surface is called "spot" or "feature". The probes are then immobilized on the surface and targets are applied in a solution after being labeled fluorescently (Ehrenreich, 2006). Probes can be sysnthetic oligonucleotides, amplicons or larger DNA fragments. There can be thousands of probes in a single microarray of 1-2 cm^2. The probes are spotted on the surface either covalently or non-covalently by a variety of techniques like inkjet deposition or photolithography. The fluorescently labeled target hybridizes with the probe, and then scanning the microarrays allows getting an image. Scanning can be done by fluorescence scanning or mass spectroscopy (Heller, 2002).

The first thing to do is to isolate the total RNA for studying gene expression by microarrays. Then RNA is reverse transcribed to cDNA which can be labeled either during or after the reverse transcription depending on the technology used. Labeling can be achieved by Cy3 and Cy5 flurophores or biotin accordingly. Under appropriate binding conditions the labeled cDNA is hybridized onto the array which occurs due to the affinity of single-stranded DNA to bind to its complementary sequence. Scanning gives signal intensities of the hybridized probes which show the expression levels of each specific gene (Dharmadi and Gonzales, 2004).

Being fast, miniaturized, automated and high-throughput, DNA microarrays are powerful tools for genome-wide gene expression studies. Among the DNA microarray systems the Affymetrix GeneChip®s are the most prominent microarrays and are widely used.

1.8.1 Affymetrix GeneChip® Platform

The GeneChip®s manufactured by Affymetrix (Santa Clara, CA, USA) contain very high feature densities that having 400,000 features on a single array is typical. The 25 residues long oligonucleotides are synthesized chemically directly on the quartz surface by photolithography, as shown in Figure 1.10. A picture of GeneChip® is shown in Figure 1.11.

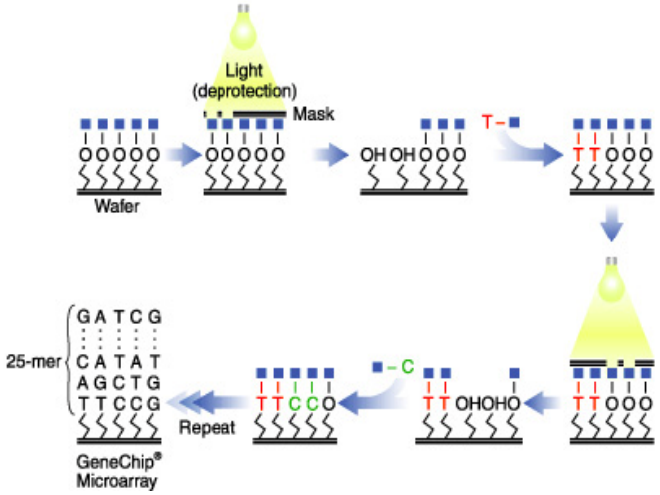


Figure 1. 10 Photolithography used for Affymetrix GeneChip® manufacturing (German Cancer Research Center, http://www.dkfz.de/gpcf/24.html. Last access date: July 01, 2011)

Figure 1. 11 A picture of Affymetrix GeneChip® cartridge (Gene Networks in Seed Development, http://seedgenenetwork.net/, Last access date: July 16, 2011)

Each probe is called “match” and has a negative control which has a single differing base in the middle of the probe which is called “mismatch probe”. Match and mismatch probes together make a “probe pair”. Cross hybridizations are detected and eliminated by the probe pairs, since cross-hybridization and the background signal can be subtracted directly from the signal from correct hybridized probes. Probe pairs of 11-15 make a “probe set” representing a single gene.

Affymetrix GeneChip® systems use a single-color detection scheme, where biotin-labeled targets hybridize with the probes and hybridizations are detected by streptavidin-phycoerythrin (Figure 1.12). This method eliminates dye effects and makes the experimental design and statistical analysis easier (Ehrenreich, 2006). Figure 1.12 shows the target preparation and labeling steps for a prokaryotic GeneChip®.

Affymetrix GeneChip®s are advantageous especially when the starting material is limited. Only 50–100 ng RNA is usually enough and there are standardized protocols appropriate for low RNA input. GeneChip®s are accepted as the optimal method for expression profile studies because of the high genetic content and high reproducibility (Hardiman, 2004).

Affymetrix offers a number of GeneChip®s already available for many eukaryotic and prokaryotic organisms. The company also manufactures custom design chips for any organism. A GeneChip® can be designed from whole genome of an organism or sequences from different organisms as well, as described in GeneChip® CustomExpress™ Array Design Guide. The researches provide the sequence in a proper format. For a prokaryotic custom design, the company and the researchers together determine the appropriate array format, feature size, mismatch strategy, probe selection region and number of probes in a probe set. There are some standard control probes in order to control whether the target preparation and the hybridization are done successfully. Depending on the array capacity, the researchers are free to add species specific controls. According to the sequence and design files

the company manufactures the probes on the arrays. They also provide a library file to be used in the data acquisition and analysis steps.

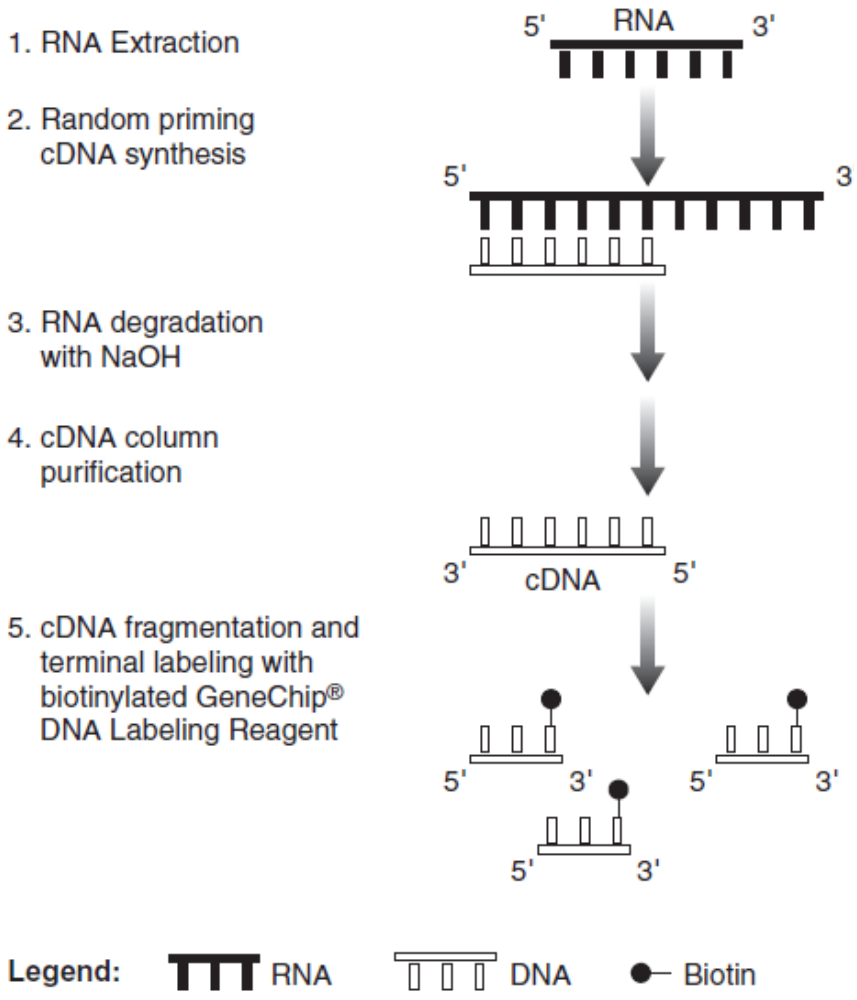


Figure 1. 12 Target labeling for prokaryotic GeneChip® antisense arrays (Affymetrix expression analysis technical manual

Up to now microarray of *Rhodobacter sphaeroides* has been designed and studied by Pappas et al. (2004). They used custom design Affymetrix GeneChip® in a similar manner with this study. Moreoever, a microarray study was carried out for *Rhodopseudomonas palustris* by Oda et al. (2005) but they had the probes

representing two copies of the genome printed on Corning UltraGAPS coated slides where the probes were printed by UV cross-linking onto the surface. A microarray study for another PNS bacterium, *Rhodospirillum rubrum* was conducted by spotting oligonucteotides on glass slides in triplicate by the manufacturer Eurogentec SA (Belgium) (Mastroleo et al., 2009).

1.9 *Rhodobacter capsulatus* Genome

The genome of *Rhodobacter capsulatus* has been sequenced (Haselkorn et al., 2001). It has a single chromosome and a circular plasmid, which are 3,738,958 bp and 132,962 bp long, respectively. Both the chromosome and the plasmid have GC rich sequences (66.6%). It is found that there are 3531 open reading frames (ORF) in chromosome and 154 ORF in the plasmid. There are 53 tRNA genes and 4 rRNA operons. The genome has 91% coding density. 3100 ORF are found to have functions and 610 ORF have similarities to hypothetical genes in databases. The remaining ORF do not have any homologues in the databases. The nucleotide sequence of *R.capsulatus* is available in GenBank with the accession numbers CP001312 for the chromosome and CP001313 for the plasmid pRCB133 (Strnad et al., 2010).

The sequence of *R.capsulatus* revealed the presence of all genes required for a complete photosystem in terms of organization and structure. The operons for light-harvesting complexes (LH I and II) and structural proteins of reaction center (B800/850 and B-870) are found to be present. Operons for bacteriochlorophyll and carotenoids are found in the genome sequence of the bacterium. All of the genes for synthesis and degredation of fatty acids (saturated and monounsaturated) have been identified. Six open reading frames belonging to sigma factors are present in the genome. The signaling and chemotaxis genes have also been identified. Enzymes taking place in carbohydrate metabolism together with the transporters for some substrates of carbohydrate metabolism such as sucrose, mannitol and lactate are found. Moreover, the genes encoding enzymes of the central carbohydrate metabolism including the glycolytic pathway and TCA cycle and glyoxylate shunt

are found in the genome. Related to the nitrogen metabolism, two nitrogenases are identified: iron-molybdenum nitrogenase and iron-iron nitrogenase. Genes of proteins in recombination, transcription, DNA repair and energy flux are found with missings in the related metabolisms.

R. sphaeroides genome was also sequenced by Zhou et al. (2003) by whole genome shotgun optical mapping. The same method was applied for the sequencing of the genome of *R. rubrum* (Reslewic et al., 2005). Moreover, the genome of the bacterium *R. palustris*, being a PNS bacterium, was sequenced (Larimer et al., 2003).

1.10 Aim of This Study

The goal of biohydrogen production studies is to produce hydrogen on industrial scale, in outdoor conditions under the natural sunlight at high efficiency. Under these conditions photofermentative hydrogen production is highly affected by fluctuated temperature. Hence, it is important to understand the temperature stress induced metabolic changes in PNS bacteria. Several studies have been conducted on the effects of temperature on hydrogen production by PNS bacteria at physiological level. However, metabolic changes induced by temperature stress have not been studied in detail at molecular level. The aim of this study is to reveal a whole geneome expression profile of *R. capsulatus* under heat and cold stress, giving emphasis on hydrogen production metabolism. For this purpose we carried out :

- Custom design of *R. capsulatus* GeneChip® which was manufactured by Affymetrix.
- Growth of bacteria under bhydrogen production conditions at control (30 °C), cold (4 °C) and heat (42 °C) stress
- Evaluation of physiological changes (pH, H_2 production, substrate conversion efficiency, yield, molar productivity, light conversion efficiency and product yield factor)

- Optimization of total RNA isolation at high purity and quality for microarray applications
- Optimization of cDNA synthesis and microarray hybridization protocols from isolated RNA samples
- Microarray analysis and data evaluation

Data obtained from this study will be useful in understanding the changes in genetic regulations under temperature stress conditions and may lead to manipulations in the bacteria in the favor of increased hydrogen yield for use in large scale industrial photobioreactors.

CHAPTER 2

MATERIALS AND METHODS

2.1 The Microorganism

Rhodobacter capsulatus DSM 1710 strain was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, German Collection of Microorganisms and Cell Cultures, Braunsweig, Germany).

2.2 Culture Media

2.2.1 Solid Medium

The bacteria form the stock (-80 °C) were activated by inoculation on a solid medium. In preparation of the solid medium the components of the rich MPYE (mineral-peptone-yeast extract) medium were dissolved in distilled water and 1.5 % agar (m/v) was added in order solidify the medium. After sterilization by autoclaving, the medium was poured into sterile plates and bacteria were inoculated on the solid medium. The composition of the MPYE medium is given in Appendix A.

2.2.2 Liquid Medium

Minimal Biebl and Pfennig (1981) medium was used with some modifications. Acetate was added as the carbon source and glutamate was added as the nitrogen source to minimal Biebl and Pfennig medium. Vitamins, trace elements and iron-citrate were also added to the medium. The compositions of the growth and hydrogen production media, vitamin, trace elements and iron-citrate are given in Appendix A.

The components of Biebl and Pfennig medium were dissolved in distilled water and acetate (20 mM) and glutamate (10 mM) were added for the preparation of growth medium. The hydrogen production medium, on the other hand, included 30 mM acetate and 2 mM glutamate. pH of the media were adjusted to 6.4 by 5 M NaOH. The medium, trace elements and iron-citrate solutions were sterilized by autoclaving. Vitamin solution containing biotin, thiamin and niacin was filter sterilized by 0.2 μm filter since high temperature causes denaturation of the vitamins.

2.3 Experimental Set-up

In this study glass bottles of 55 ml volume were used as photobioreactors. The bacteria were inoculated into the liquid growth medium to make the culture 50ml. The bottles were sealed with sterile rubber taps. Both the glass bottles and the ruber taps were sterilized in the autoclave (Nüve OT 032 autoclave). Then the photobioreactors were placed in a cooling incubator (Nüve ES 250 cooled incubator) in order to keep the temperature constant. The inner culture temperatures were measured by a digital thermometer (Maxi-T). Illumination was provided by the use of 100 Watt incandescent lamps and the light intensity was adjusted to 2000 lux by a lux meter (Lutron LX-105 Light Meter).

The evolved gas was collected by gas collection tubes which were filled with distilled water and closed by a rubber tap. The air flow was prevented by sealing the rubber tap with parafilm. The gas collection tubes and the reactor bottles were connected to each other through plastic tubings having needles at both ends. Air flow was prevented on the tubings by teflon bands and parafilm. The evolved gas was collected by the water displacement method (Uyar et al., 2007). The schematic diagram and the photograph regarding the experimental set-up are shown in Figure 2.1 and Figure 2.2, respectively.

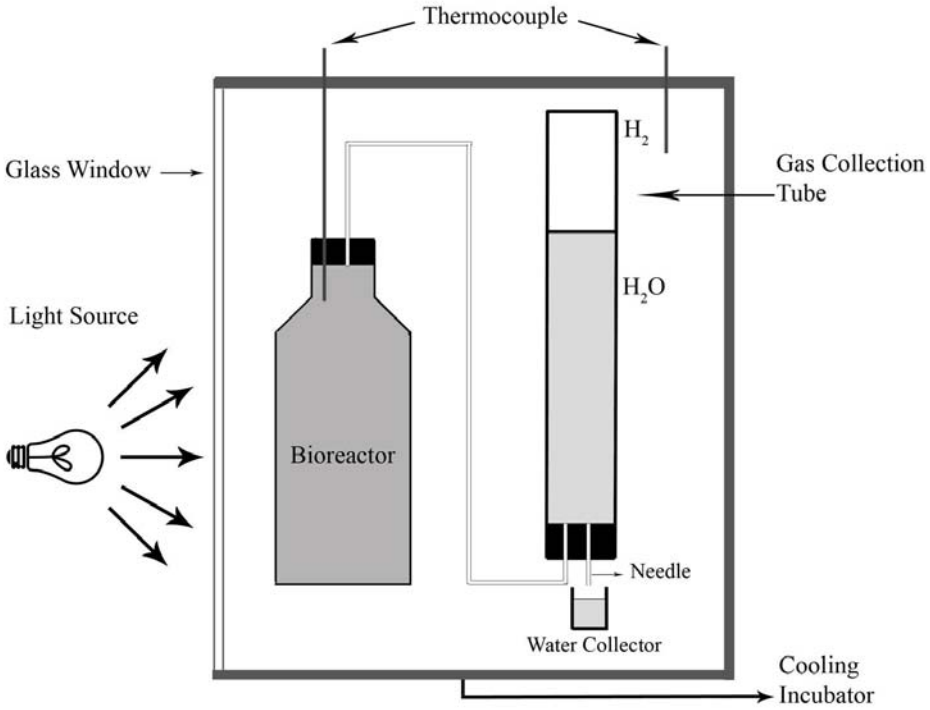


Figure 2. 1 Schematic diagram of the experimental set-up (Sevinç, 2010)

Figure 2. 2 The photograph of the experimental set-up

2.3.1 Activation and Growth of Bacteria

The bacterial stocks which were in 30% glycerol and stored at -80 ºC had to be activated first. In order to activate the bacteria from the stock, they were inoculated onto agar plates containing the solid medium under sterile conditions by streak plate method. The plates were then incubated at 30 ºC sealed with alliminum foils to provide darkness in the cooling incubator. After about one week a single colony was picked and inoculated into 1 ml sterile growth medium (20 mM acetate/10 mM glutamate). The volume of the bacterial culture was increased up to 50 mL by passaging the bacteria into fresh growth medium each time when the optical density (OD_{660nm}) was around 1.0.

2.3.2 Experimental Set-up for Hydrogen Production

After the bacteria became active enough they were inoculated into hydrogen production medium (30 mM acetate/2 mM glutamate) to have a starting OD_{660nm} of 0.230-0.250 in 50 ml of total culture. The photobioreactors were then sparged with argon gas (>99% purity) for 3 minutes to provide an anaerobic environment. These steps were all carried out under sterile conditions. Then the bottles were placed in the cooling incubator with the gas collection tubes connected to the bottles.

2.4 Analyses

Liquid samples were removed from the reactor bottles every day in order to measure optical density, pH and organic acid consumption. Equal amount of sterile distilled water was given into the culture to balance the pressure in the reactor bottles. The volume of the produced gas was determined by water displacement method (Figure 2.1) and the volume of hydrogen was calculated from the percentage in the total gas produced. The ideal gas law formula PV = nRT was used to calculate the millimoles of hydrogen produced.

2.4.1 Cell concentration

The cell concentration of the cultures in reactor bottles was measured by a spectrophotometer (Shimadzu UV-1201) at a wavelength of 660nm. Distilled water

was used as the blank because 10 times dilution was done on the bacterial culture for the optical density measurement. The calibration curve was used to convert the absorbance values to dry cell weight values (Uyar, 2008). The calibration curve of dry cell weight versus OD_{660nm} is given in Appendix B.

2.4.2 Gas Composition Analysis

Gas samples were taken from top of the reactor bottles by gas-tight syringe (Hamilton, 22 GA 500μL gas tight No. 1750). The composition of the produced gas was determined by a gas chromatograph (GC) (Agilent Technologies 6890N) in which the column used was Supelco Carboxen 1010. The chromatograph had a thermal conductivity detector. The carrier gas was argon with a flow rate of 26 ml/min. The oven temperature was 140 °C and the temperatures of injector and detector were 160 °C and 170 °C, respectively. The software used was Agilent Chemstation ver.B.01.01 (Agilent Technologies). A sample gas chromatogram is given in Appendix C.

2.4.3 pH Analysis

pH analysis of the cultures were done by the use of a pH meter (Mettler Toledo 3311). The pH meter was calibrated with pH 7 and pH 4 solutions before use.

2.4.4 Organic Acid Analysis

The daily taken bacterial culture samples were centrifuged at 13,600 rpm for 10 minutes in a bench-top centrifuge (Eppendorf MiniSpin) to precipitate the cells. The supernatant was used to measure the organic acid concentrations. The supernatant was filtered with 45 μm nylon filters (Millipore, 13 mm) to remove impurities. Then the samples were analyzed by High Performance Liquid Chromatography (HPLC)

equipped with Alltech IOA-1000 (300mm ×7.8 mm) column. 10 µL of sample was injected into the system by the autosampler (Shimadzu SIL-20AC). The UV detector (Shimadzu SPD-20A) detected the organic acids at 210 nm wavelength. The oven temperature was kept at 66 °C. The mobile phase was 0.085 M H_2SO_4 with a flow rate of 0.4 ml/min with a low gradient pump (Schimadzu LC-20AT).

The calibration curves of the analyzed organic acids, namely acetic acid, lactic acid, formic acid, butric acid and propionic acid, were constructed for different concentrations of pure organic acids. Calibration curves were used for the determination of the concentrations of the organic acids. A sample HPLC chromatogram and a sample calibration curve are given in Appendix D.

2.5 Microarray Analysis

2.5.1 Design of Affymetrix GeneChip®

Since a microarray chip for *Rhodobacter capsulatus* was not present in Affymetrix catalog, the GeneChip® Custom Array was designed according to the GeneChip® Custom Expression Array Desgin Guide. The whole genome sequence of *R.capsulatus* was taken from http://rhodo.img.cas.cz (Strnadl et al., 2010).

A sequence file in FASTA format, an instruction file containing the name, the product, role and description of the probe sets with the name of the genes, and a design request form was prepared and sent to Affymetrix Custom Array Design Team.

The GeneChip® Custom Arrays for *R. capsulatus* were prokaryotic antisense DNA arrays. The feature size was 11 micron and the format was 100-3660. The probes were selected from 600 bases from 3' end of the gene and intergenic region sequences. 13 probe pairs were produced for each sequence by match/mismatch probe strategy, i.e. a mismatch probe having a single base mismatch in its center was generated. All sequences were pruned against all other sequences. Totally 4052 probe sets were present on the array.

The arrays had quality control and alignment controls along with the target

preparation and hybridization controls. Quality control and alignment controls are the grid alignment corner checkerboards, the text on the array (the array name) (Appendix G), edge controls, borders, quality control oligo controls and the center block. The target preparation controls that were tiled on the arrays were Poly-A Controls which are *dap*, *lys*, *phe*, *thr*, *trp* from *B. subtilis*. Hybridization controls consisted of *bioB*, *bioC*, *bioD* from *E.coli* and *cre* form P1 bacteriophage.

2.5.2 Temperature Stress Application and Sample Collection

In order to isolate total RNA form the bacterial culture, a similar procedure to the hydrogen production experiment was carried out except that the photobioreactors' volumes were 150 ml. The volume was increased because large amount of bacterial culture had to be removed from the photobioreactors for the RNA isolation at different times.

The photobioreactors having bacteria in hydrogen producing medium were incubated at 30 °C for 48 hours. After 48 hours three bottles were passed to 4 °C and three bottles were passed to 42 °C in other two cooling incubators (Nüve ES 250 cooled incubator). The remaining three bottles were kept at 30 °C to be the control condition. The experimental procedure is shortly shown in Figure 2.3.

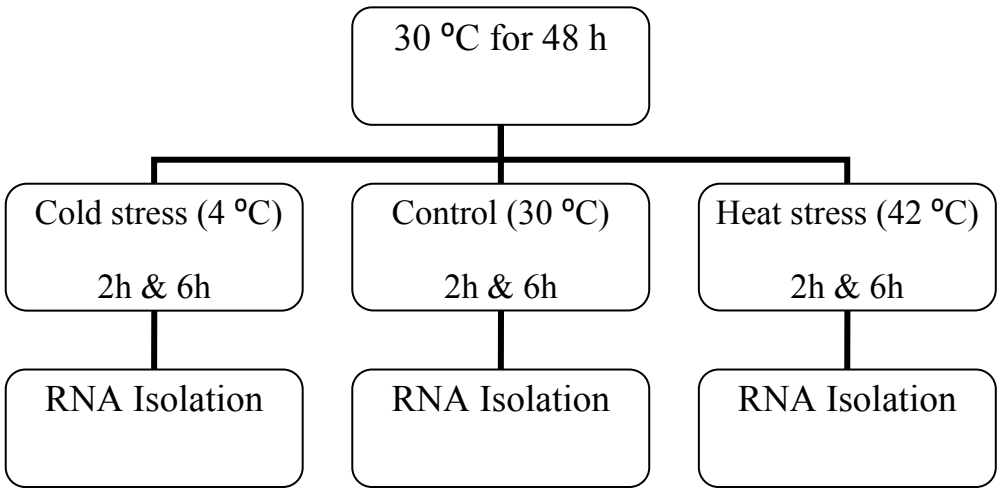


Figure 2. 3 Experimental procedure for stress application and sampling for RNA isolation

At the time of stress (0 hour) and 2, 6, and 12 hours after the stress the optical density of the cultures were measured and appropriate amount of bacterial cultures to be equal to $1x10^9$ cells/ml were put into 2 ml microcentrifuge tubes under sterile conditions. Then these microcentrifuge tubes were centrifuged at 12,000g for 15 min at 4 °C, the supernatants were removed and the microcentrifuge tubes with bacterial pellet were inserted into liquid nitrogen and stored at -80 °C until the time of RNA isolation.

2.5.3 RNA Isolation and Characterization

The bacterial cell pellets that were kept at -80 °C were dissolved in 200 µl of 5 mg/ml lysozyme solution (Sigma, activity ≥ 40,000 units/mg protein) by vortexing for 10 seconds in order to lyse the cells. The lysozyme solution was prepared in 1X Tris EDTA (TE) Buffer. The composition of TE buffer is given in Appendix E. Then the samples were incubated at 37 °C for 10 min in a heater block (WTW-CR 3200). In order to degrade the proteins in the samples 20 µl of 3 mg/ml. Proteinase K solution (AppliChem, activity 37.5 m Anson U) was added on the lysed cells and incubated at 60 °C for 10 min. 1 ml of TRIzol® Reagent (Invitrogen) was added on the samples and mixed by vortexing for 1 minute, then the samples were kept at room temperature for 5 minutes. 200 µl chloroform (Merck) was added onto the samples and mixed by vigorous shaking for 15 seconds. The samples were kept at room temperature for 6 minutes and centrifuged at 12,000g for 15 minutes at 4 °C in a cooling centrifuge (Sigma 4K15). After this step, 3 phases occurred in the microcentrifuge tubes. The most upper clear phase contained the RNA, the middle phase contained the DNA and the bottom phase contained the TRIzol® Reagent, cell debris and proteins. 650 µl of the upper phase was collected into a new microcentriguge tube. Then 500 µl of cold isopropanol was added and mixed by inverting 40 times and kept at room temperature for 10 minutes. The microcentrifuge tubes were then centrifuged at 17,000g for 10 minutes at 4 °C. At this step RNA formed a clear pellet at the bottom of the microcentrifuge tubes, so the supernatant was removed and the pellet was washed with 1 ml of 80% ethanol (Merck) by

vortexing and centrifugation at 20,000g for 10 minutes at 4 °C. The supernatant was removed and the white pellet which is RNA was let for air dry for a few minutes. After the remaining ethanol was dried, the RNA pellet was dissolved in 20 µl of RNase free ultra pure water (MilliQ) by incubating at 60 °C for 10 minutes. RNA solution was then kept at -80 °C for further use.

For microarray studies a large amount of RNA with high purity is required. The concentration of total RNA was measured by a micro-volume spectrophotometer (NanoDrop 2000) at 260 nm wavelength using 1 µl of total RNA. Since protein contamination would be a problem, the measurement at 280 nm is also important and the ratio of 260/280 gives the purity of the RNA. The 260/280 ratio should be ≥ 1.80 for microarray studies (Dumur et al., 2004). The concentrations of the isolated RNA from bacterial samples are given in Table 3.1. A sample NanoDrop profile is given in Appendix F.

The isolated RNA should be intact and away from genomic DNA contamination. In order to determine the integrity of RNA, 1 µL of total RNA was loaded into a gel-based chip (RNA 6000 NanoChip) and analyzed by Agilent 2100 Bioanalyzer. cDNA synthesis

The steps in the Affymetrix Expression Analysis Technical Manual for Prokaryotic Target Preparation were followed with some minor modifications for the preparation of cDNA to be hybridized onto the chips.

First of all, 10µg of total RNA was mixed with 10 µl of 100 ng/µl random primers (Invitrogen) in an RNase free microcentrifuge tube. 2 µl of poly-A RNA controls containing polyadenylated transcripts for *B.subtilis* genes was added with nuclease free water (Affymetrix) to make up the total volume to 30 µl. The composition of the poly-A RNA controls are given in Table 2.1. The RNA/Primer mix was incubated at 70 °C for 10 minutes, 25 °C for 10 minutes and then was chilled to 4 °C in a thermal cycler (Applied Biosystems, 96-well GeneAmp® PCR System 9700). After this

incubation, the RNA/Primer mix (30 µl) was mixed with 7.5 µl 200 U/µl SuperScript II™ Reverse Transcriptase and 12 µl of its buffer which is 5X first strand buffer (Invitrogen), 6 µL 100mM DTT (Affymetrix), 3 µl 10mM dNTP (Invitrogen) and 1.5 µl 20U/µl SUPERase·In™. The total 60 µl solution was incubated at 25 °C for 10 minutes, 37 °C for 60 minutes, 42 °C for 60 minutes and 70 °C for 10 minutes, then was chilled to 4 °C.

Table 2. 1 The composition of Poly-A RNA controls

Polyadenylated transcript	**Stock Concentration**	**Final concentration**
lys	7.6 nM	0.256 pM
phe	15.2 nM	0.511 pM
thr	30.4 nM	1.022 pM
dap	114.0 nM	3.833 pM

2.5.4 Removal of RNA

After the synthesis of cDNA on RNA, the RNA should be degraded to yield the cDNA. In order to remove RNA, 20 µl of 1N NaOH (Merck) was added on cDNA/RNA solution and incubated at 65 °C for 30 minutes. Then, 20 µl 1N HCl (Merck) was added in order to neutralize NaOH.

2.5.5 Purification and Quantification of cDNA

MinElute PCR Purification Kit (QIAGEN) was used to purify the single-stranded cDNA. Firstly, the cDNA solution (100 µl) was mixed with 500 µl of Buffer PB, the binding buffer. The mixture was vortexed and applied on the MinElute PCR

Purification columns (QIAGEN). The column was centrifuged at 17,920g for 1 minute at 20 °C. The flow-through was discarded; the column and the collection tube were reused. 750 µl of washing buffer, Buffer PE, was added on the column and centrifuges at 17,920g for 1 minute at 20 °C. The flow-through was discarded and the column with the collection tube was centrifuged at 17,920g for additional 1 minute at 20 °C. The column was put in a new clean 1.5 ml microcentrifuge tube. 12 µl of the elution buffer was added on the column and incubated at room temperature for 1 minute. The column and the microcentrifuge tube were centrifuged at 17,920g for 1 minute at 20 °C. The flow-through contained single-stranded cDNA in 11 µl elution buffer.

The concentration of the cDNA in 1 µl was measured by micro-volume spectrophotometer (AlphaSpec™) at 260nm. At least 1.5 µg cDNA was needed to continue to the next step and to obtain sufficient amount of material to hybridize onto the microarray chip.

2.5.6 cDNA Fragmentation

For the fragmentation reaction, 20 µL of reaction mix was prepared containing 10 µl cDNA, 2 µl 10X DNase Buffer (Ambion), DNase I (Ambion, 2U/µl) and nuclease free water (Affymetrix). The amount of DNase I was calculated to be 0.6 U DNase I for 1 µg of cDNA. The reaction mix was incubated at 37 °C for 10 minutes. Then DNase I was inactivated at 98 °C for 10 minutes.

2.5.7 Terminal Labelling

The fragmentation products were labeled at the 3' termini by biotin in the labeling reaction. The reaction mix was prepared by 20 µl of fragmented cDNA products, 10 µl 5X Reaction Buffer (Promega), 2 µl 7.5mM GeneChip® DNA Labelling Reagent (Affymetrix), 2 µl Terminal Deoxynucleotidy Transferase (Promega, 30 U/µl) and 16 µl nuclease free water (Affymetrix). The reaction mix was incubated at 37 °C for 60 minutes. After that the reaction was stopped by adding 2 µl 0.5M EDTA (Invitrogen, pH 8).

2.5.8 Hybridization

For 100 Format array, 130 µl of hybridization cocktail was prepared in order to hybridize the fragmented and labeled cDNA onto the array. 1.5 µg of cDNA was used for hybridization. 2.2 µl 3nM Control Oligo B2, 65 µl 2X hybridization mix, 10.2 µL DMSO, 6.5 µl 20X Hybridization Control and nuclease-free water (volume was determined according to the volume of cDNA).

The GeneChip® Probe Array was let to equilibrate to room temperature to inhibit any cracks that would occur on the septa during the loading of the hybridization cocktail. A clean pipette tip was inserted into the upper septum in order to ventilate the array chamber so that the hybridization cocktail could be loaded easily. 130 µl hybridization cocktail is loaded into the other septum with a micropipette. The GeneChip® Probe Array and the septa are shown in Figure 2.4.

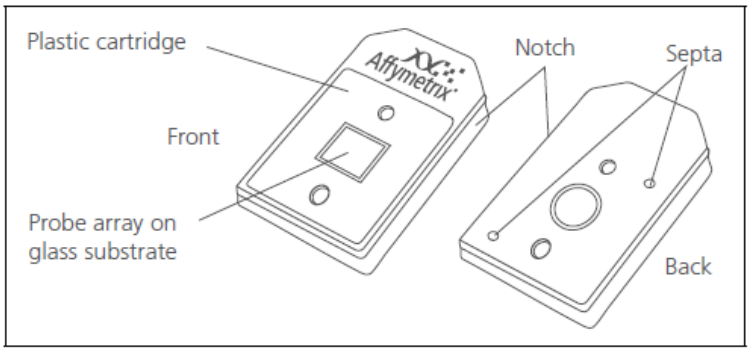


Figure 2. 4 GeneChip® Probe Array

Rhodobacter capsulatus have a high GC content (66.6%). Because of this reason the hybridization temperature and washing and staining protocol used for *P. aeruginosa* were used, since *P. aeruginosa* had high GC content, too. The microarray chips were incubated at 50 ºC with rotating at 60 rpm at GeneChip® Hybridization Oven 640 for 17 hours.

2.5.9 Washing and Staining

After the hybridization step, the chips were washed and stained at GeneChip® Fluidics Station 450. Firstly, the experiment name, probe array type, sample name, sample type and project were determined in GeneChip® Operating Software (GCOS), since the fluidics station was operated using GCOS. The buffers Wash A and Wash B and distilled water were placed into the place determined for them. Moreover,

Stain Cocktail 1, Stain Cocktail 2 and Array holding buffer were aliquoted into microcentrifuge vials in volumes of 600 µl, 600 µl and 800 µl, respectively, and placed into the pre-determined holders on the fluidics station. Arrays were then placed into the appropriate modules. The fluidics station was then run with the fluidics protocol Flex FS450_0002.

2.5.10 Scanning

After washing and staining, Tough-Spots were applied to the back of the arrays in order to prevent any leakage from the array cartridge. Then they were scanned using GeneChip® Scanner 3000, which was operated by GCOS.

2.5.11 Data Analysis

After the scanning of the chips, GCOS acquires data in files that are supported by some other analysis programs like GeneSpring. Each array consists of 4 files in .EXP, .CHP, .DAT, .CEL formats, which contain raw image data needed for data analysis. .EXP file contain experimental information like the project name, sample name, array type. .DAT file contains the chip image after the scanning. The .DAT file should be inspected for any image artifacts like scratchers, bubbles, etc. A sample .DAT file image is given in Appendix G. The computerized version of .DAT file is the .CEL file which is used to generate the .CHP file containing the signal intensity values of the probes. One another file is the .RPT file is the report file having information about the quality criteria like the controls' intensities, the average background and noise intensity, percent of the present and absent signals, etc. In order to analyze the data, .CHP files of the arrays were loaded to GeneSpring GX 11.

The technology that was generated by our custom design chip was named as Affymetrix.GeneChip®.TR_RCH2a520699F. The arrays were compared and statistically analyzed and the results are shown in sections 3.7-3.16.

CHAPTER 3

RESULTS AND DISCUSSIONS

In this study effects of temperature on hydrogen production metabolism of *R.capsulatus* were investigated by physiological approaches and microarray analysis. Bacterial cell growth, hydrogen production, organic acid (acetate) consumption, pH change under temperature stress conditions were determined. RNA isolation from *R.capsulatus* was optimized. Microarray studies were carried out by using custom designed Affymetrix GeneChip®. The gene expression changes of the bacterium under cold (4 °C) and heat stress (42 °C) was analyzed comparing with control condition (30 °C).

3.1 Effect of Temperature Stress on Growth of *R.capsulatus*

Bacterial cell growth of *R.capsulatus* under different temperatures (4 °C, 30 °C, 42 °C) is given in Figure 3.1.

Mesophiles have the optimum growth rate at between 20 °C and 40 °C. R.capsulatus, being a mesophile, can grow well in this range. When the temperature exceeds this range, the growth of the bacteria ceases and they enter death phase, as seen from the Figure 3.1. Since high temperatures cause denaturation of the proteins, the enzymatic reactions required for growth and other metabolisms may not occur. In the present study, the incubator temperature was set to 42 °C but the culture temperature increased to 43-44 °C due to the illumination. The culture temperature under cold stress , when the incubator temperature was 4 °C, was 7-8 °C, and it did not decrease even when the incubator was set to 0 °C in one trial. The bacteria under cold stress showed faster growth than the optimum condition. In a study by Corner and Kotrola

(1995), survival of *E.coli* at 4 ºC was enhanced when organic acids like acetic, citric and lactic acid were present in the medium compared to the medium without organic acids. The presence of acetic acid in the present study may have led this growth pattern of *R.capsulatus* under cold stress.

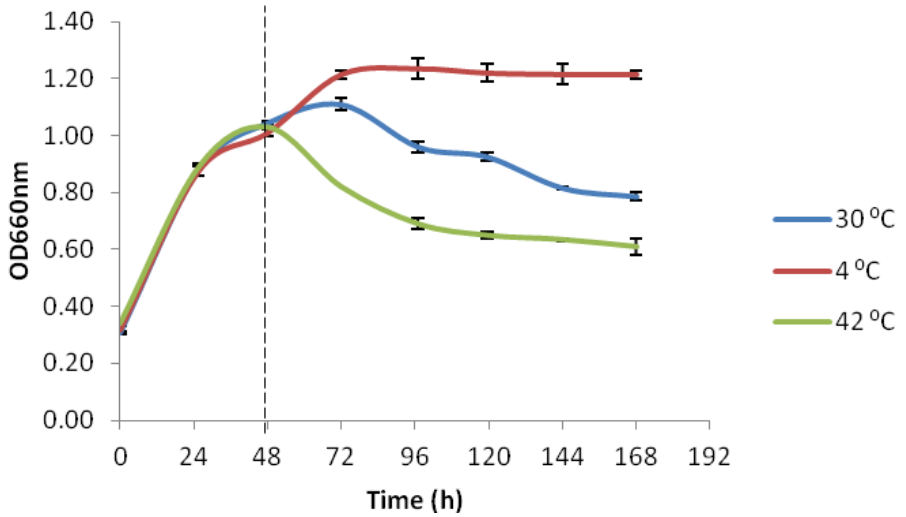


Figure 3. 1 Bacterial cell growth under temperature stress in hydrogen production medium The dash line indicates the time of stress application

The initial cell concentration was 0.17-0.19 gdcw/L (0.310-0.350 OD_{660nm}) as seen in Figure 3.1. The lag phase was not observed because the cell concentration was high enough to skip the lag phase in hydrogen production medium. Usually bacteria have a generation (doubling) time changing in between 15 minutes to 24 hours. In the present study, the doubling time of *R.capsulatus* in the exponential phase under 30 ºC, 2000 lux illumination with 30 mM acetate in the medium was about 14.5 hours. Environmental conditions like temperature, pH or nutrient sources change the generation time. Bacteria stop increase in number due to lack of nutrient or accumulation of wastes in stationary phase where their cell sizes decrease since less nutrient is needed for the maintenance of smaller cells. In order to protect DNA and proteins bacteria produce enzymes to resist stress in this phase. In the death phase the cells die. The rate of death is hard to determine because some mutations may arise

for better survival or some cell may grow by cannibalizing others (Slonczewski and Foster, 2009).

3.2 Effect of Temperature Stress on pH

The Figure 3.2 shows the change in the pH of the medium under heat, cold stress and control conditions. Up to the 48th hour, the time of the start of the stress (shown by dash line in the graph), the pH increased, but stayed in the range suitable for hydrogen production. This increase was due to the consumption of acetic acid in the medium. Heat stress did not affect pH significantly, however under cold stress pH increased up to 7.423. Under heat stress the consumption of acetic acid ceased (Section 3.3), but in cold stressed samples all the remaining acetic acid was consumed, which may increase pH.

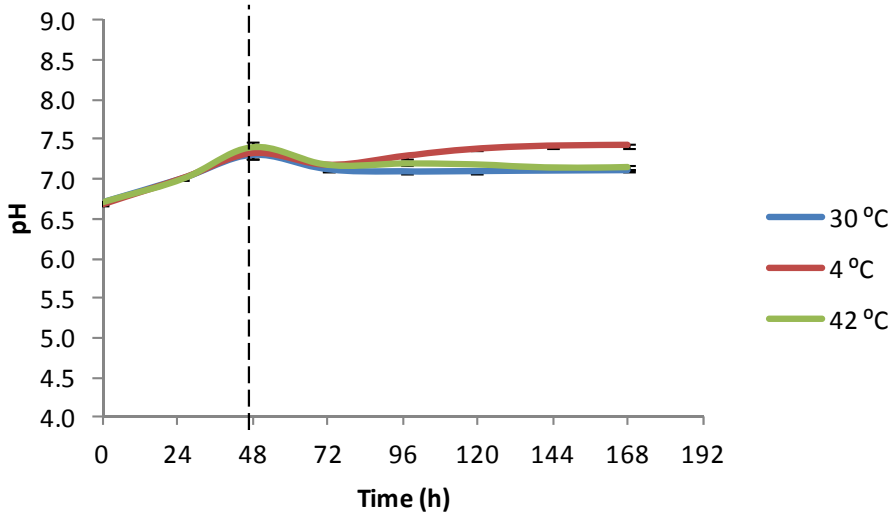


Figure 3. 2 pH change under temperature stress in hydrogen production medium. The dash line indicates the time of stress application

3.3 Effect of Temperature Stress on Utilization of Acetate

The starting concentration of acetate was 30 mM which was totally consumed under optimum temperature and cold stress. However, 5 mM acetate remained in the medium under heat stress. One of the major effects of heat stress is on the cell

membrane which may affect uptake of substrates. The ratio of unsaturated to saturated fatty acyl chains become lower with increasing temperature in *E.coli* and many other bacteria increasing the fluidity of membrane (Mejía et al., 1995). The inability of *R.capsulatus* cells to uptake acetate under heat stress may be a result of membrane damage upon high temperature. Membrane fluidity is also affected by cold stress thus substrate uptake may be slowed down (Phadtare, 2004). As seen from Figure 3.3 the rate of acetate consumption under cold stress was slower than that of the optimum temperature. In the section 3.5, the substrate conversion efficiencies are calculated and shown in Table 3.1.

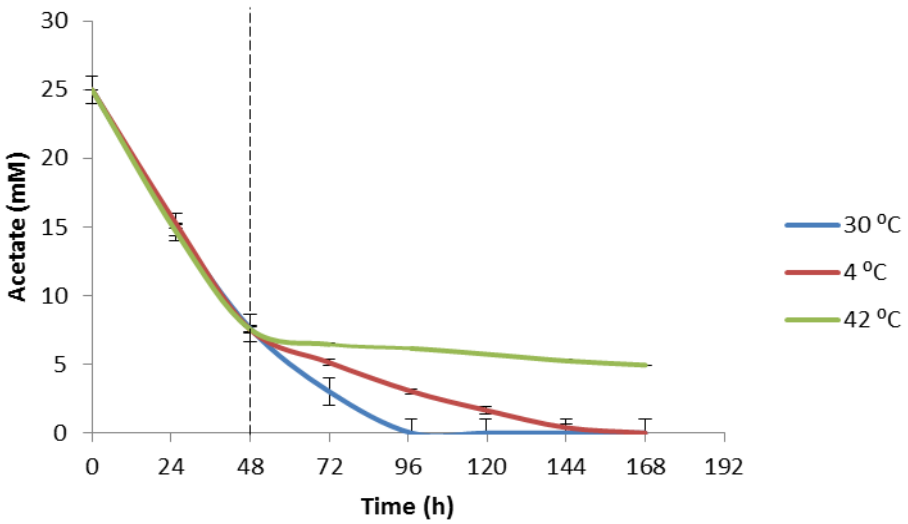


Figure 3. 3 Acetate consumption under temperature stress in hydrogen production medium. The dash line indicates the time of stress application

3.4 Effect of Temperature Stress on Hydrogen Production

The cumulative hydrogen production in millimoles was plotted against time and shown in Figure 3.4. The mole of hydrogen was calculated according to the following formula of ideal gas law:

$$PV = nRT \tag{3.1}$$

In this formula P is the atmospheric pressure and taken as 1 atm, V is the volume of produced hydrogen in L, n is the mole of produced hydrogen. R is molar gas constant which is equal to 0.082 $L.atm.mol^{-1}.K^{-1}$. The temperature, T, is taken in Kelvin. The amount of hydrogen in the produced gas was determined by gas chromatography. 85% of the produced gas was hydrogen in all cases and 15 % was found to be CO_2.

Hydrogen production of *R.capsulatus* stopped under both cold and heat stress. Since hydrogen production occurs by enzyme catalyzed reactions, decreased or no hydrogen production beyond the optimum temperature was expected.

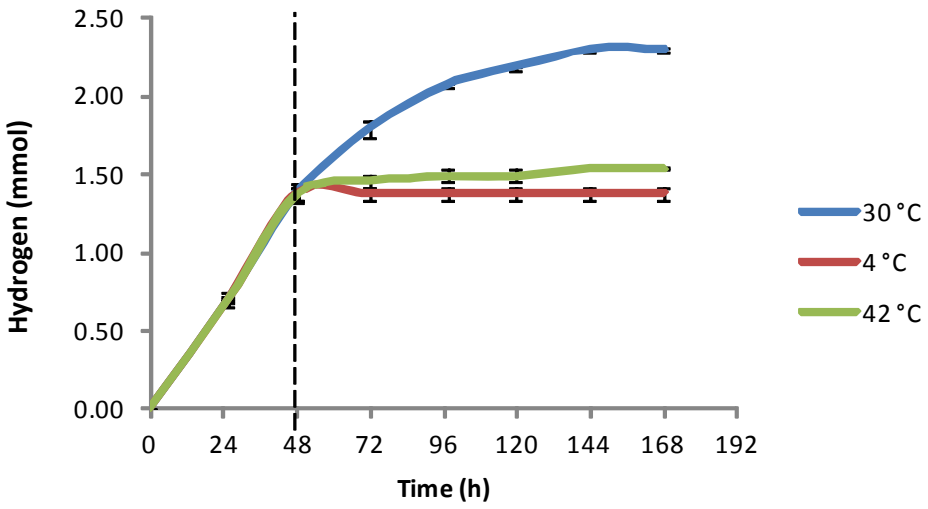


Figure 3. 4 Cumulative hydrogen production under temperature stress in hydrogen production medium. The dash line indicates the time of stress application

In the study of Zhang and Shen (2006), the effect of temperature on hydrogen production of mixed culture of anaerobic bacteria by dark fermentation was investigated. They found that optimal temprature of hydrogen production by the mixed bacteria possesing nitrogenase and hydrogenase systems was 35 °C, and at 45 °C hydrogen production significantly decreased. The authors concluded that this decrease was because of the non-optimal temperature for enzyme activity.

3.5 Calculations of Substrate Conversion Efficiency, Yield, Molar Productivity, Light Conversion Efficiency and Product Yield Factor

There are some important parameters in analysis of hydrogen production. Besides cumulative amount of produced hydrogen, substrate converison eficiency, yield, molar productivity, light converison efficiency and molar yield factor should be taken into account in hydrogen produciton analysis. These parameters are calculated in this section.

Substrate conversion efficiency is defined as follows:

$$\frac{\text{number of moles of hydrogen produced}}{\text{stoichiometric number of moles of hydrogen that would be produced from full use of the initial substrates}} \times 100 \quad (3.2)$$

In this study, the substrate for hydrogen production was acetate and initial concentration of acetate was 30 mM. The reaction of hydrogen production from acetate is:

$$C_2H_4O_2 + 2H_2O \rightarrow 4\ H_2 + 2\ CO_2 \quad (3.3)$$

Based on the stoichiometry of the above reaction, substrate conversion efficiencies of the bacteria under control, cold stress and heat stress were calculated according to the experimental data and shown in Table 3.1. Since the initial acetate amounts were equal and hydrogen production is highest under control condition, substrate conversion efficiency was also highest under this condition. Conversion of acetate into hydrogen under both stress conditions was close to each other and lower than

that of control. It was found by He et al. (2006) that substrate conversion efficiency of *R.capsulatus* was maximum at 30 °C compared to 26 °C and 34 °C.

Yield, as given in Equation 3.3, is the ratio of moles of hydrogen produced to the moles of the substrate utilized, in this case acetate. Theoretical yield of H_2 for 1 mole of acetate is 4, as given in Equation 3.2. Yield was calculated according to the experimental data and shown in Table 3.1.

$$\text{Yield} = \frac{\text{Number of moles of hydrogen produced}}{\text{Number of moles of substrate utilized (mol)}} \quad (3.4)$$

All of the acetate was consumed in control and cold stress conditions as shown in Figure 3.3, but there were about 5 mM acetate left in the medium under heat stress. Similar to substrate converison efficiencies, yields of stress conditions were lower than control condition.

Molar productivity is defined as:

$$\frac{\text{cumulative millimoles of hydrogen produced}}{\text{volume of culture (L)} * \text{t(hour)}} \quad (3.5)$$

The duration of hydrogen production from the end of lag phase to the end of hydrogen production is shown by t in the above formula. Therefore, molar productivity of hydrogen production is molar rate of this production. In the present study, molar productivities were calculated for the time before stress application (before 48 h) and after application of stress (after 48 h). As seen from Table 3.1, H_2 produciton stopped with cold stress, since there was a great decrease in temperature, from 30 °C to 4 °C. The rate of hydrogen production under heat stress was approximately 8 times slower than that under optimum temperature. Eroğlu et al.

(2010) concluded that highest hydrogen productivity of *R.capsulatus* was accomplished at 30 °C.

Light converison efficiency, η, is the ratio of the obtained total energy of hydrogen to the total energy input to the photobioreactor by light radiation. It is calculated by the formula:

$$\eta\,(\%) = \frac{33.6 \times \rho_{H_2} \times V_{H_2}}{I \times A \times t} \times 100 \qquad (3.6)$$

The constant number 33.6 is the energy density of hydrogen gas in Watt.h/g. The density of hydrogen shown by ρ_{H2} is 0.089 g/L. V_{H2} shows the volume of produced hydrogen in L. The photobioreactors were under constant illumination of 2000 lux in this study, hence the light intensity, I, is equal to 114.286 (Watt/m^2). A in the formula is the irradiated area and it is 0.002 m^2 for 55 mL photobioreactor. t is the duratioın of hydrogen production. While calculating light converison efficiencies, incident light intensity was used, not the absorbed light intensity, because the operation was in batch mode where the cell concentrations and accordingly the absorbed light intensities varied thoughout the process (Uyar et al., 2007). Under the control condition the light converison efficiency was found to be the highest, whilst it was higher under heat stress than under cold stress. After the 48th hour which was the start of stress application, the light converison efficiency under cold stress condition was 0, while it was 0.039 under heat stress, almost 6 times lower than that under control condition.

Product yield factor is another important parameter for hydrogen production analysis. It is cumulative hydrogen production in millimoles divided by maximum dry cell weight in grams. Product yield factor can be a measure of relative values of cell growth and hydrogen production. This is important because for both hydrogen production and cell growth the same substrate is used. Therefore, it can be concluded

that higher cell growth is not desirable in terms of hydrogen production. Koku et al. (2002) suggested the use of substrates favorable for hydrogen production, which would not let excessive cell growth. From this point of view, higher product yield

factor may be considered as a consequece of utilization of higher amount of substrate for hydrogen production. Product yield factors were calculated and shown in Table 3.1. Similar to the other parameters, product yield factor under control condition was the highest followed by that under heat stress and finally the cold stress.

Table 3. 1 Molar productivity, substrate conversion efficiency, yield, light converison efficiency, and product yield factor values for control, cold stress and heat stress conditions

	Total H_2 Produced (mmol)	Molar productivity (mmol/L.h)		Substrate conversion efficiency (%)	Yield ($mol_{H2}/mol_{acetate}$)	Light conversion efficiency (%)	Product yield factor (mmol H_2/gdw)
		Before 48 h	After 48 h				
Control (30°C)	2.29	0.574	0.230	44.4	1.775	0.400	75.02
Cold Stress (4 °C)	1.38	0.574	0	26.7	1.077	0.242	40.63
Heat stress (42 °C)	1.54	0.574	0.029	29.8	1.430	0.269	54.37

In microarray studies the optimization of RNA iolation from *R.capsulatus* was achieved. Microarray analysis was performed to compare gene expression profiles of *R.capsulatus* under cold and hest stress conditions.

3.6 Microarray Analysis

3.6.1 RNA Isolation and Characterization

Affymetrix Expression Analysis Technical Manual includes the protocols for both eukaryotic and prokaryotic arrays. The required total RNA concentration for prokaryotic arrays which will in turn be used in cDNA synthesis is 10 µg corresponding to minimum 555 ng/µl according to the reaction volume (18 µl). In a previous gene expression study with *R. sphaeroides*, Akköse et al. (2009) used TRI REAGENT (Sigma) for total RNA isolation from 10-15 ml of bacterial culture and used 1 µg total RNA treated with DNase for reverse transcriptase-polymerase chain reaction. This volume of starting bacterial culture for 1 µg total RNA is high. Moreover, it has long been known that DNase treatment degrades also the RNA (Maxwell et al., 1976). For this reason, in the present study RNA isolation without any DNase treatment was aimed because for prokaryotic microarray experiments besides high purity of total RNA, high amount is also needed. Manufactured RNA isolation kits result in DNA-free RNA and they have been frequently used for total RNA isolation in expression studies. RNeasy Mini Kit (QIAGEN) and RiboPure–Bacteria Kit (Ambion) were used in RNA isolation from *R.capsulatus* in this study. DNA-free RNA was obtained; however, the concentrations were not higher than 150 ng/µL. Because of the unsatisfactory results form tried RNA isolation kits, manual isolations using TRIzol (Invitrogen) were carried out. During these isolations mainly the protocol of TRIzol Max Bacterial RNA Isolation Kit and the protocol of Chomczynski and Sacchi (1987) were followed, but some modifications were done in order to increase the yield and purity of isolated RNA. First of all, starting bacterial cell amount was increased to $1x10^9$ cells and higher RNA yield was achieved. Different form the manual, lysozyme treatment was carried out. *R.capsulatus* possesses capsule in the cell envelope, which should be broken down for extracting RNA. Briiutigam et al. (1988) showed that lysozyme enzyme broke down the capsule of this bacterium. In a study conducted to increase RNA yield of *B.subtilis*, Guez et al. (2009) tried lysozyme and zirconia beads and showed that 10 minutes of lysozyme incubation together with agitation gave the highest RNA yield,

which was also applied in this study. Use of proteinase K resulted in increase in purity. Modifications are done on some of the centrifugation steps, like increasing time and centrifugation speed. For biological samples with low RNA yields some researchers pool RNA samples before hybridization. Pooling may also be used to decrease biological variation among the samples and this technique allows to decrase

the number of arrays to be used, thus decreasing the cost (Shih et al., 2004). Peng et al. (2003) studied the effects of pooling on biological variations and differential expressions and concluded that pooling is statistically valid and efficient. On the other hand, in a technical note by Affymetrix, pooling was said to decrease sensitivity and increase false positives. Pooling was tested in this study; however the RNA yield decreased significantly after pooling procedure, therefore pooling of RNA samples were not carried out in the present study.

The concentration of total RNA samples isolated were determined by using NanoDrop and are shown in Table 3.2.The optical density ratio of 260/280 is a measure of the RNA quality, since it determines the purity; the presence or absence of contaminations like protein contamination. The RNA having the 260/280 optical density ratio greater than 1.8 is accepted as RNA of good quality (Fleige and Pfaffl, 2006). Since all the RNA samples are of good quality, they were used for microarray experiments and hybridizations on microarray chips, scanning, image acquisition and data analysis were carried out.

The integrity and quality of total RNA samples were also checked using Agilent 2100 Bioanalyzer. The electrophoresis gel image and electropherograms revelaed that all isolated RNA samples were intact and free from DNA contamination, therefore they were good for use in microarray experiments (Figure 3.5 and 3.6).

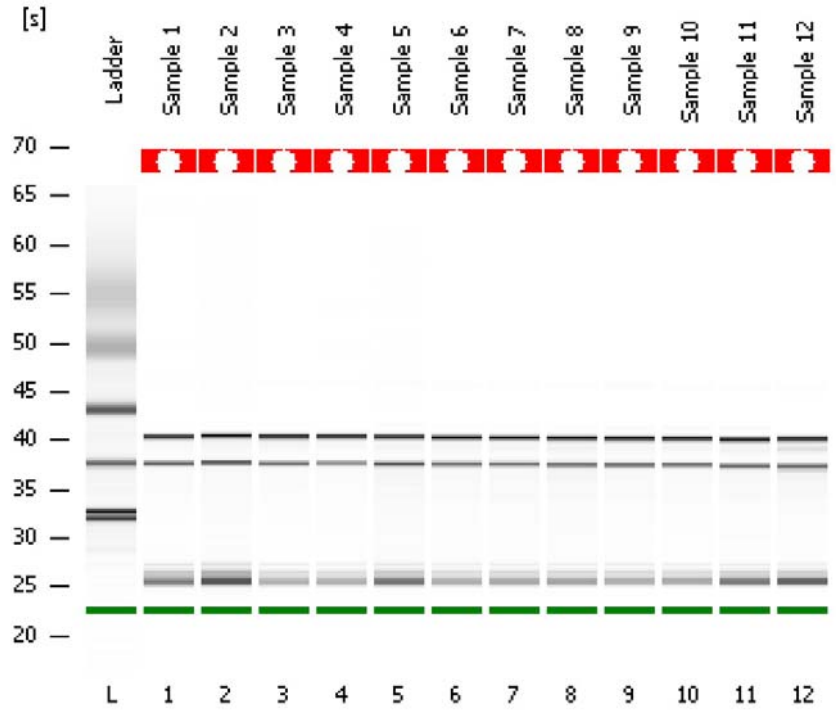


Figure 3. 5 Bioanalyzer gel picture for total RNA of *Rhodobacter capsulatus*. Line at 25th s indicates 5S rRNA, line at 38th s indicates 16S rRNA and line at 40th s indicates 23S rRNA.

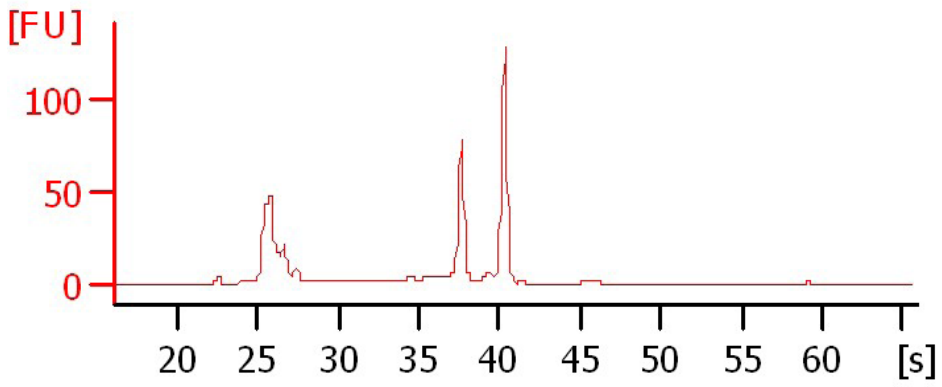


Figure 3. 6 Bioanalyzer electropherogram for total RNA of *Rhodobacter capsulatus*. Peak at 25th s indicates 5S rRNA, peak at 38th s indicates 16S rRNA and peak at 40th s indicates 23S rRNA.

Table 3.2 The concentrations and purity (260/280) of *R.capsulatus* total RNA. Experiments were carries out in triplicates

	0 hour		2 hours		6 hours	
	Concentration (ng/µl)	260/280	Concentration (ng/µl)	260/280	Concentration (ng/µl)	260/280
Control (30°C)	661.2	1.89	744.8	1.87	1035.8	1.9
	745.2	1.92	708.2	1.95	840.6	1.91
	739	1.79	738.4	1.81	764.9	1.79
Cold Stress (4°C)	787.8	1.88	806.6	1.94	740	1.98
	1138.2	1.89	1138.2	1.81	943.2	1.97
	562.8	1.87	597.8	1.86	522.7	1.9
Heat stress (42°C)	1006.8	1.95	960.2	1.96	923.8	1.94
	907	1.87	862	1.9	1005.4	1.93
	650.4	1.86	633.4	1.88	744.8	1.87

3.6.2 Microarray Data Quality Control

As defined in Section 2.10 Design of Affytmerix GeneChip®, the files after chip scanning in GCOS program were obtained. The .DAT files of arrays were inspected for any image artifacts and no abnormalities were observed in any chips. A sample .DAT file image is given in Appendix G. The other quality control parameters on the chip image, alternating intensity pattern on the borders, checkerboards on the corners and the array name were alright for all the chips and a sample for these parameters is given in Appendix G. Moreover, the report files (.RPT file) containing the average background, noise, percent present values, poly-A and hybridization controls were analyzed for the similarities between the sample groups. According to the report files of the scanned microarray chips, one replicate of each sample were found to be significantly different in terms of the average background, noise and percent present values than the other replicates. One reason for this situation may be that the cDNA synthesis and hybridization experiments of the single replicates were carried out on a

different day, approximately 4 months later than the others. Dumur et al. (2004) studied on the quality control criteria and emphasized on the day-to-day effect by saying that even slight differences in the composition of the solutions and pipetting errors might affect the noise values. Hybridization controls were significantly affected from day-to-day effect in their study. Though, they also concluded that microarray platforms like Affymetrix can be very useful for gene expression studies, since the variability in data was due to some exogenous reasons.

The chips of those single replicates were not used in data analysis, since it was recommended by Affymetrix that the compared arrays should have comparable background, noise and percent present. The other chips were similar to each other in terms of the quality control parameters, so data from them were analyzed using GeneSpring analysis program. The values of quality control parameters for the selected chips are given in Table 3.3, which was generated according to the study of Yilmaz, Yücel and Öktem (2008).

GeneSpring GX 11 was used for the analysis of GeneChip® microarray data. First of all, the .CEL files were loaded to the program and projects of cold and heat stress were created. Advanced workflow was followed instead of default guided workflow of the program, since guided workflow performed first significance analysis then fold change analysis, which resulted in a very small entity list. Following advanced workflow, experiments were created choosing appropriate samples, which were data files different from raw hybridization data files because samples were created in GeneSpring with the appropriate technology specific to the organisms and chips. Then, the samples were grouped as, for example, cold stress (2h), cold stress (6h) and control (30 °C), accordingly and interpretations were formed like cold stress and heat stress, separately. Quality control analyses on the samples were done and they are shown in the related sections below. Fold change analysis was constructed and entity lists of fold change ≥ 2.0 were formed, on which significance analyses were done. Since there were 3 groups (cold stress (2h), cold stress (6h) and control) within an interpretation One-way ANOVA was performed.

Table 3. 3 The values of the quality control criteria probe sets of selected chips

Chip Name	Average Background	Noise	AFFX-Lys Sig	AFFX-Phe Sig	AFFX-Thr Sig	AFFX-Dap Sig	AFFX-BioB Sig	AFFX-BioC Sig	AFFX-BioD Sig	AFFX-cre Sig	% Present
30°C 0h-1	63.79	5.79	P (0.22)	P (0.48)	P (0.80)	P (0.71)	P (1.62)	P (0.56)	P (1.58)	P (0.9)	46.0
30°C 0h-2	81.38	7.46	P (0.32)	P (0.51)	P (0.79)	P (0.68)	P (1.48)	P (0.55)	P (1.56)	P (0.92)	50.0
4°C 2h-1	44.21	2.21	P (0.34)	P (0.61)	P (0.92)	P (1.02)	P (1.81)	P (0.56)	P (1.76)	P (0.92)	70.6
4°C 2h-2	53.38	2.96	P (0.30)	P (0.61)	P (0.98)	P (1.02)	P (1.65)	P (0.59)	P (1.62)	P (0.89)	65.1
4°C 6h-1	103.26	10.39	P (0.32)	P (0.52)	P (0.83)	P (0.74)	P (1.40)	P (0.62)	P (1.39)	P (0.93)	57.1
4°C 6h-2	98.46	8.97	P (0.19)	P (0.57)	P (0.94)	P (0.68)	P (1.25)	P (0.60)	P (1.60)	P (0.88)	42.9
42°C 2h-1	47.34	2.82	P (0.38)	P (0.46)	P (0.95)	P (0.87)	P (1.65)	P (0.53)	P (1.69)	P (0.93)	73.8
42°C 2h-2	50.79	2.94	P (0.46)	P (0.43)	P (0.85)	P (1.00)	P (1.67)	P (0.60)	P (1.81)	P (094)	68.1
42°C 6h-1	77.06	6.92	P (0.45)	P (0.54)	P (0.82)	P (0.70)	P (1.35)	P (0.58)	P (1.54)	P (0.90)	40.1
42°C 6h-2	83.04	9.86	P (0.22)	P (0.55)	P (0.86)	P (0.71)	P (1.31)	P (0.61)	P (1.56)	P (0.92)	52.3

In the scope of MicroArray Quality Control (MAQC) Project Shi et al. (2008) inspected the reproducibility of microarray data and concluded that discordance between the differential gene expression lists might result from analysis solely by statistical significance (p) using t-tests and the more stringent the p-value threshold, the less reproducible the differential gene expression lists. When the ranking criterion is fold change with a non-stringent p-value cut-off, the gene lists become more reproducible. That is why in the present study fold change ≥ 2.0 followed by a significance analysis with p-value cut-off 0.1 were chosen.

3.6.2.1 Cold stress vs. control

In order to analyze the effects of cold stress on *R. capsulatus* with respect to the optimum growth and hydrogen production conditions, data from the scanned chips were grouped in GeneSpring. Before going on with the significance analysis the samples were inspected if they pass the quality control criteria analysis on the chips were carried out. The correlation coefficients calculated by Pearson correlation for each pair of arrays are shown in Figure 3.7.

Array Name	0 h 30-1.CEL	0 h 30-2.CEL	6 h 4-1.CEL	6 h 4-2.CEL	2 h 4- 1.CEL	2 h 4- 2.CEL	Cold Stress
0 h 30-1.CEL	1.0	0.9742222	0.9519176	0.9649935	0.82934517	0.8572263	Control (30°C)
0 h 30-2.CEL	0.9742222	1.0	0.96643746	0.98530155	0.8729336	0.9003339	Control (30°C)
6 h 4-1.CEL	0.9519176	0.96643746	1.0	0.9849007	0.9155422	0.9072018	Cold Stress (6h)
6 h 4-2.CEL	0.9649935	0.98530155	0.9849007	1.0	0.9000022	0.9197164	Cold Stress (6h)
2 h 4- 1.CEL	0.82934517	0.8729336	0.9155422	0.9000022	1.0	0.9606503	Cold Stress (2h)
2 h 4- 2.CEL	0.8572263	0.9003339	0.9072018	0.9197164	0.9606503	1.0	Cold Stress (2h)

Figure 3. 7 Correlation coefficient matrice of cold stress (2h and 6h) vs control

The correlation coefficients of the array pairs were high and close to each other, so all these arrays were used for further analysis. Indeed, fold change filtering followed by a significance analysis with one-way ANOVA ($p \leq 0.1$) was carried out on the interpretation consulted with these samples. As a result, 328 genes' expression out of 4052 probes showed statistically significant change when $p \leq 0.1$. The profile plot of

these changes of gene expression plotted with normalized intensity values are shown in Figure 3.8.

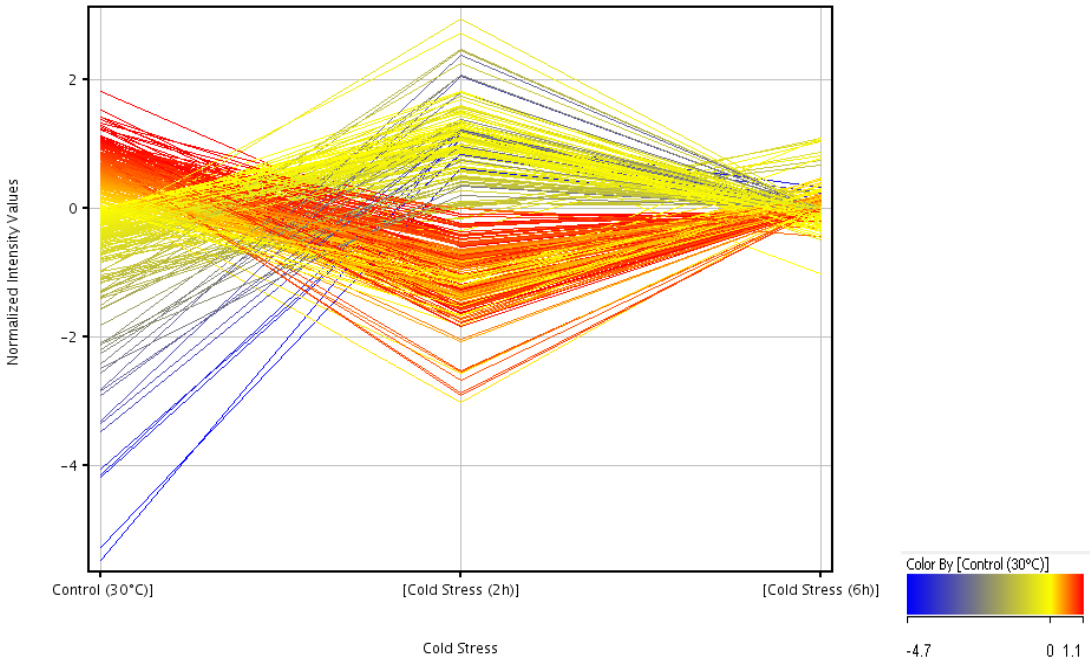


Figure 3. 8 Profile plot of cold stress (2h and 6h) vs control

The profile plot indicates that after 2h of cold stress application, the change in gene expression was higher than that after 6h with respect to the control condition. This was expected, since in bacteria the temperature stress causes rapid changes in the gene expression followed by adaptation. Adaptation of bacteria occurs in a relatively small time. Schumann (2001) pointed out that microorganisms have to adapt rapidly to environmental changes in order to survive and change in the temperature is a situation to be adapted. Beales (2004) emphasizes on the importance of adaptation to changes in temperature which has impact on all cellular reactions. In a study it was shown that *E.coli* cells showed adaptation at 4 ºC when kept at this temperature for 3 hours (Mihoub et al., 2003). Similarly adaptation of *E. coli* cells to 10 ºC in 6 hours prior to freezing was shown by Berry and Foegeding (1997). Similar results were obtained by the microarray data from this study. Comparing the scatter plots (Figure 3.9 and 3.10) drawn after fold change and significance analysis ($p \leq 0.1$), it can be

seen that most of the significantly up or down regulated genes under 2 hours of cold stress did not have significant expression changes under 6 hours of cold stress. This results revealed that after 6 hours of cold stress application, the bacterial cells showed adaptation.

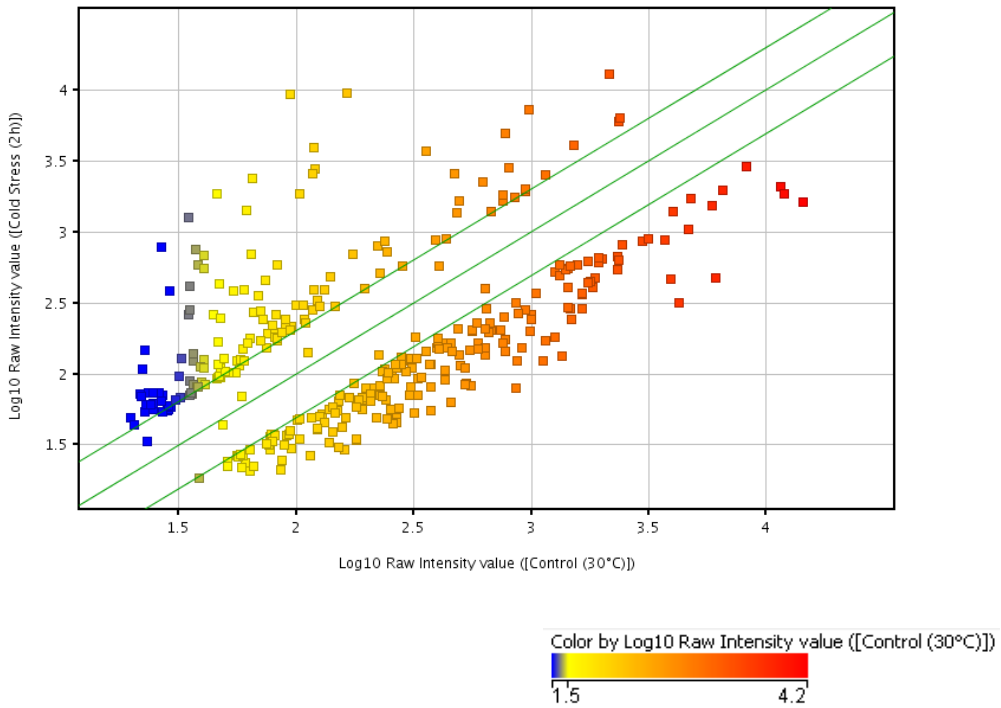


Figure 3. 9 Scatter plot of cold stress 2h vs control

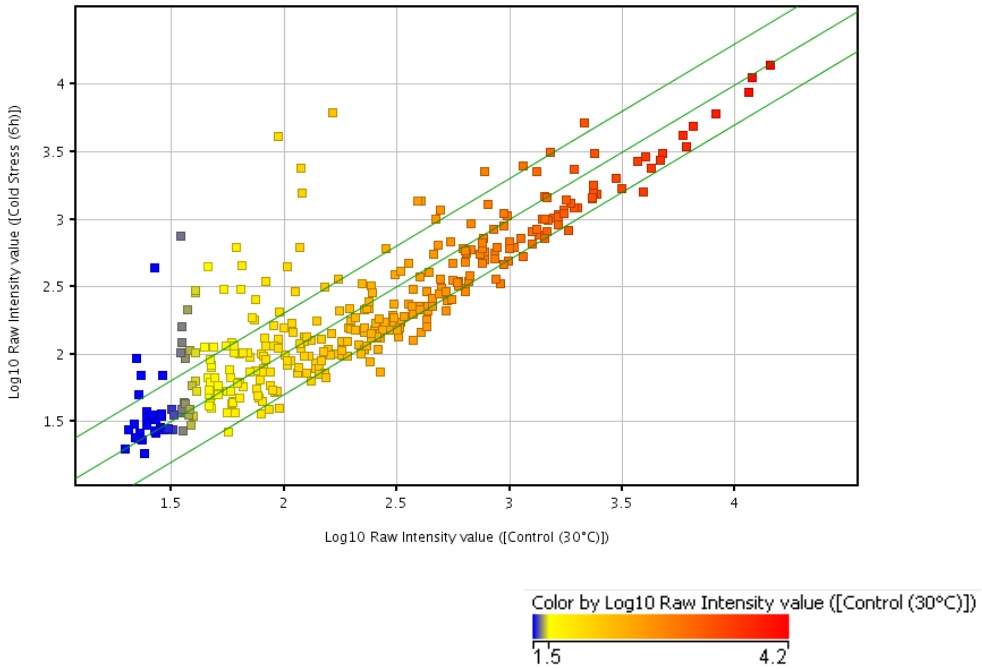


Figure 3. 10 Scatter plot of cold stress 6h vs control

3.6.2.2 Heat stress vs control

In order to analyze the effects of heat stress on *R. capsulatus* with respect to the optimum growth and hydrogen production conditions, data from the scanned chips were grouped in GeneSpring, similar to the procedure done for analysis of cold stress. Samples were analyzed in terms of the quality control parameters. The correlation coefficients calculated by Pearson correlation for each pair of arrays are shown in Figure 3.11. The correlation coefficients of the array pairs were high and close to each other, so all these arrays were used for further analysis.

Array Name	2 h 42- 1.CEL	2 h 42- 2.CEL	0 h 30-1.CEL	0 h 30-2.CEL	6 h 42-1.CEL	6 h 42-2.CEL	Heet Stress
2 h 42- 1.CEL	1.0	0.9651744	0.92097586	0.94062454	0.97186685	0.94591707	Heat Stress (2h)
2 h 42- 2.CEL	0.9651744	1.0	0.90602577	0.9198543	0.96672875	0.95779616	Heat Stress (2h)
0 h 30-1.CEL	0.92097586	0.90602577	1.0	0.97334653	0.93265754	0.93874365	Control (30°C)
0 h 30-2.CEL	0.94062454	0.9198543	0.97334653	1.0	0.9409767	0.9436911	Control (30°C)
6 h 42-1.CEL	0.97186685	0.96672875	0.93265754	0.9409767	1.0	0.9869541	Heat Stress (6h)
6 h 42-2.CEL	0.94591707	0.95779616	0.93874365	0.9436911	0.9869541	1.0	Heat Stress (6h)

Figure 3. 11 Correlation coefficient matrice of heat stress (2h and 6h) vs control

After fulfillment of the quality control criteira, fold change filtering followed by a significance analysis with one-way ANOVA ($p \leq 0.1$) was carried out on the interpretation consulted with these samples. As a result, 293 genes' expression out of 4052 probes showed statistically significant change when $p \leq 0.1$. The profile plot of these changes of gene expression plotted with normalized intensity values are shown in Figure 3.12.

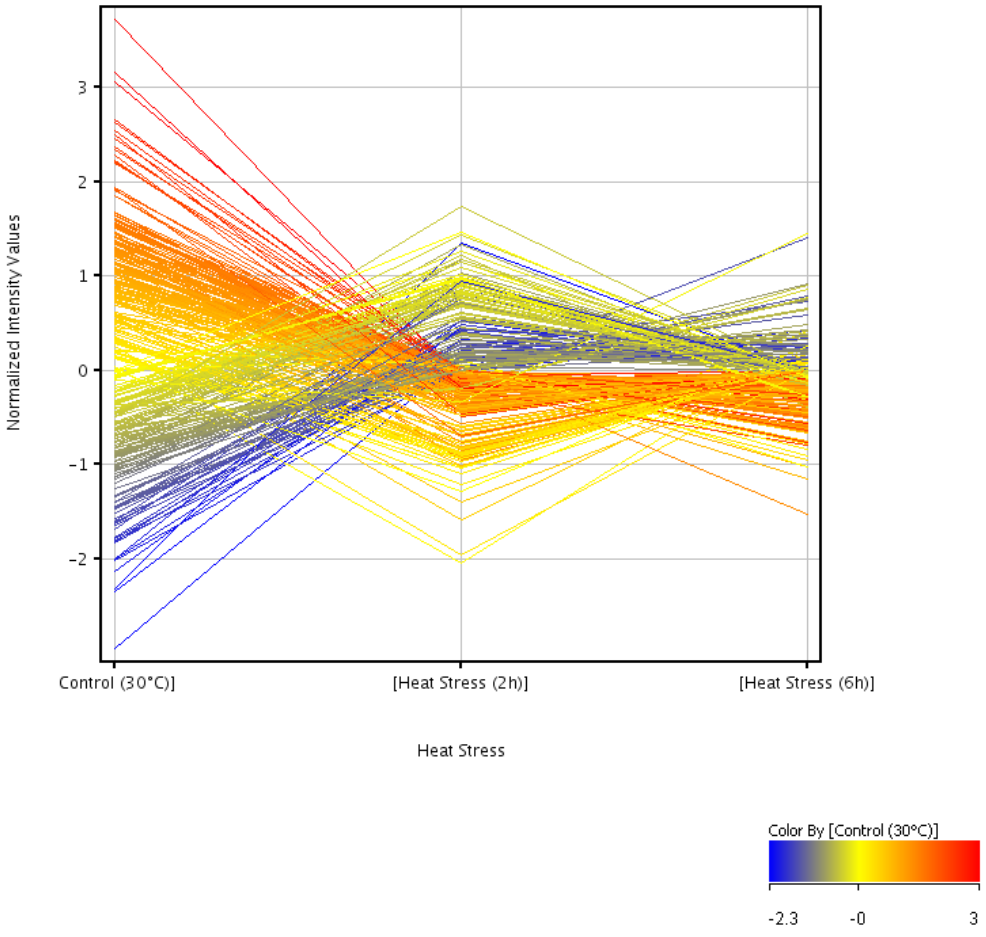


Figure 3. 12 Profile plot of heat stress (2h and 6h) vs control

The changes in the expression levels of the genes after 2 hours of heat stress were highly different than that of control condition. Similarly the expression levels of most genes after 6 hours of heat stress showed great differences compared to the control

but they were similar to that after 2 hours of heat stress. Similar conclusions can be drawn from the scatter plots in Figure 3.13 and Figure 3.14. Some genes' expression levels decreased to the level of control condition after 6h of heat stress application, while most stayed similar to that of 2h of heat stress.

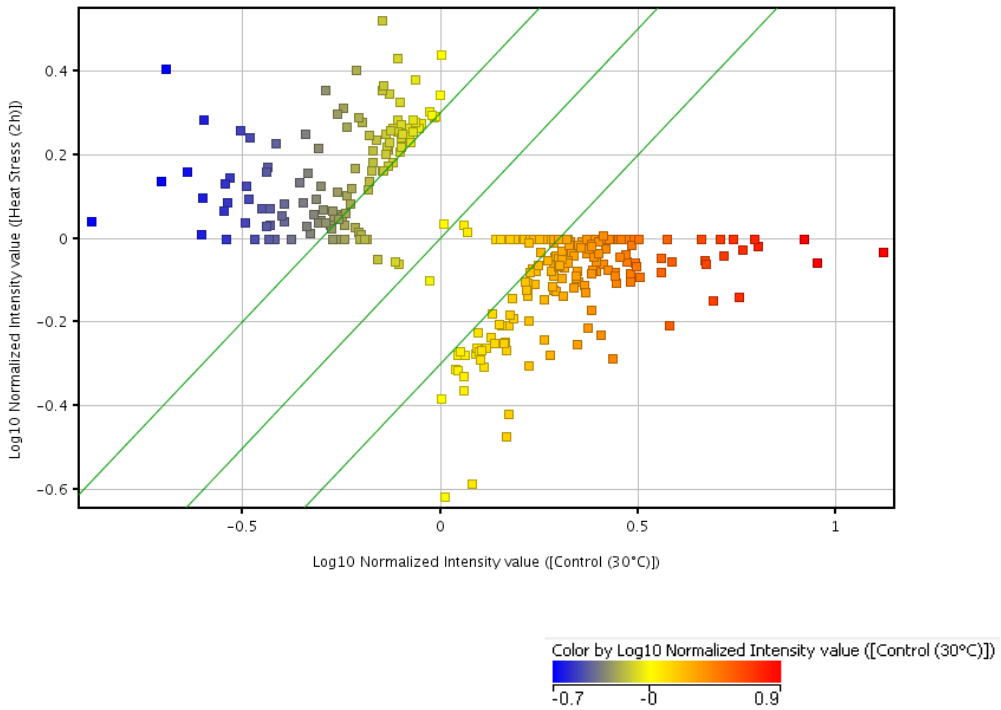


Figure 3. 13 Scatter plot of heat stress 2h vs control

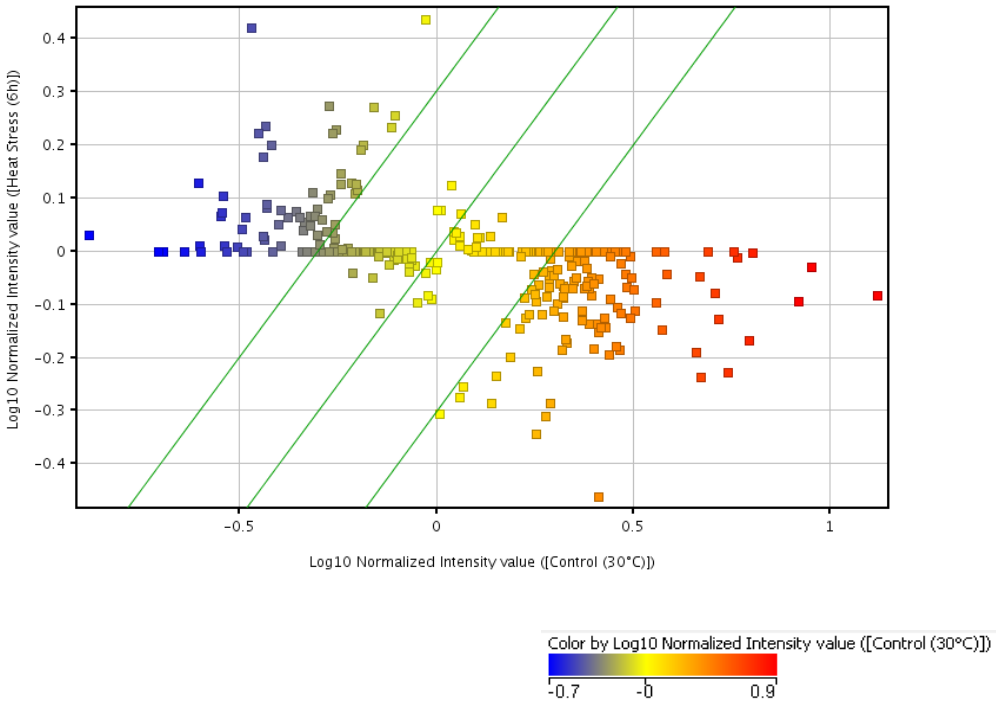


Figure 3. 14 Scatter plot of heat stress 6h vs control

Under heat stress both in bacteria and eukaryotes, level of transcription of heat shock proteins is induced. Since heat shock proteins take place in cellular repair in the molecular level, the induction of transcription of the related genes is very rapid and transient. The induction is achieved within several minutes followed by a gradual decrase to a new steady state (Yura, 2001). An example to this concept is the study of Arsène et al. (2000), where temperature shift from 30 °C to 42 °C resulted in rapid increase in the expression of heat shock proteins, followed by adaptation period in which the expression of heat shock proteins decreased to a new steady state in *E.coli*.

3.7 Gene expression analysis under temperature stress

Comparing the pie charts (Figure 3.15 and 3.16) of the distributions of the genes with significant expressional change under cold and heat stress, the nitrogen, photosynthesis metabolisms and electron transport draw attention among the other metabolisms having a dramatic change between the two stresses. Actually nitrogen

metabolism, photosynthesis and electron transport have a high importance in hydrogen production by *R. capsulatus*, indeed photoproduction of hydrogen is primarily connected with nitrogen metabolism in purple non-sulfur bacteria (Hillmer and Gest, 1977). Some genes take role in more than one metabolism, such as nitrogen metabolism and electron transport, or electron transport and photosynthesis at the same time.

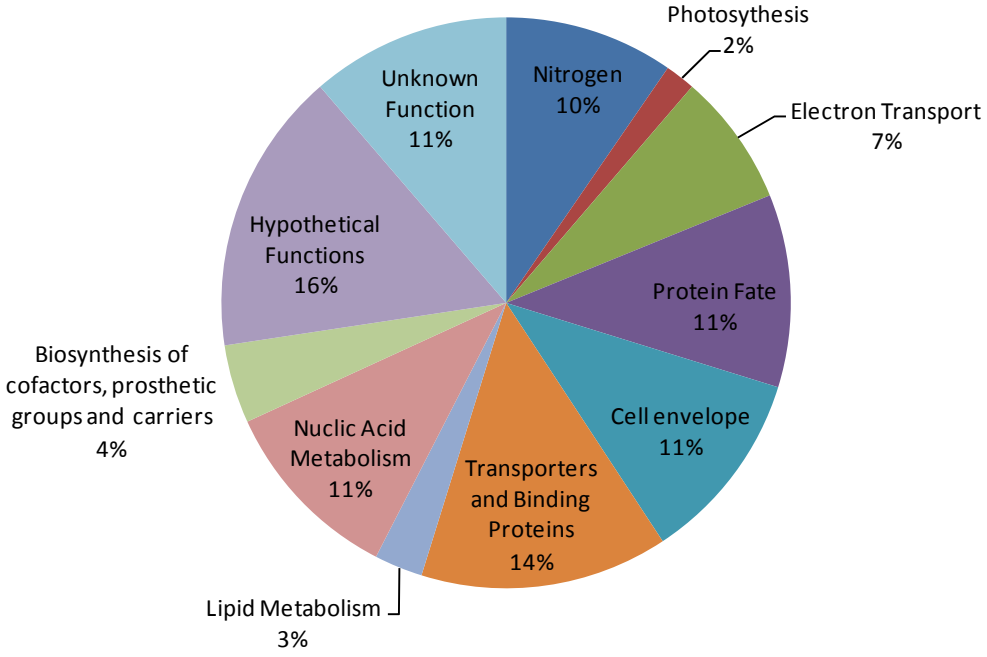


Figure 3.15 Metabolic distributions of the genes showing significant change in expression levels under cold stress according to their functions

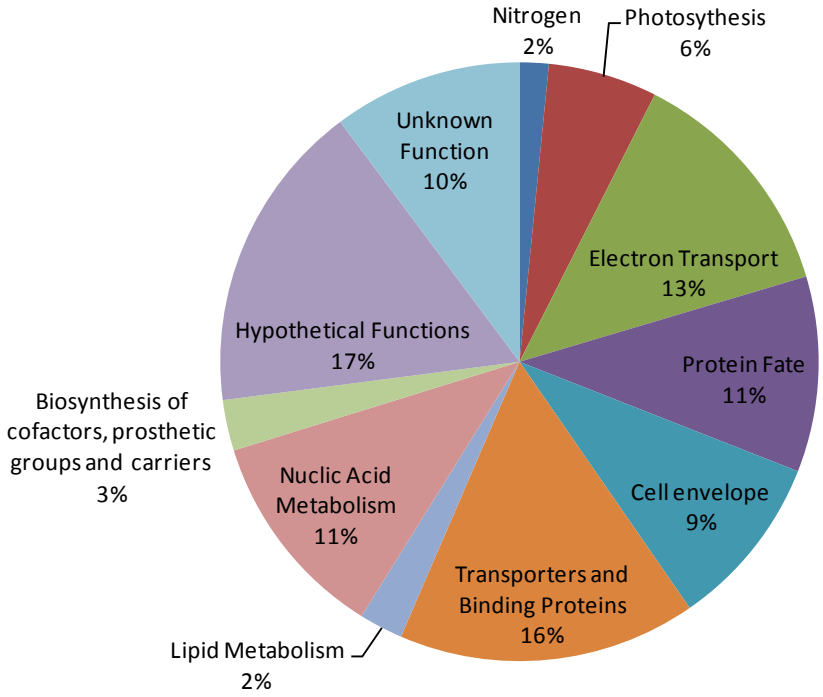


Figure 3. 16 Metabolic distributions of the genes showing significant change in expression levels under heat stress according to their functions

In the following sections the effect of cold and heat stress on different metabolisms were inspected in the light of the gene lists given in Appendices I and J. In the web site of genome sequence of *R. capsulatus* (http://onco.img.cas.cz/rhodo/results/index.html) some gene groups were generated like nitrogen genes, photosynthesis genes and electron transport genes. These gene groups together with the grouping of proteins of *R.capsulatus* by Onder et al. (2010) were used in a proper combination in this study. Some genes could not be named during the sequencing, and the functions of some genes could not be determined exactly but the metabolism they take place could be attained, for example, a gene with unknown function was suggested to be a transporter and binding protein having role in transport of aminoacids and amines.

Graphs were plotted for the genes whose expressions were significantly changed in a comparison manner. While determining the number of transcripts in these graphs,

gene expression with a fold change higher than 2.0 were chosen from the gene lists in the Appendices H and I.

It should be noted here that since microarray technologies are high throughput, they do not give exact expression levels as do the quantitative PCR methods.

3.7.1 Effect of temperature stress on nitrogen metabolism of *R.capsulatus*

Nitrogen metabolism is of vital importance as nitrogenase enzyme is employed in hydrogen production in *R. capsulatus*. As clearly seen from Figure 3.17, nitrogen metabolism was affected dramatically under cold stress, while it was not affected much under heat stress. The genes whose expressions were significantly increased under cold stress include molybedunum containing *nif*-encoded nitrogenase and *anf*-encoded iron-only alternative nitrogenase genes (Table 3.4). Significant increase in expressions of *nifB, nifD, nifE, nifH, nifK* and *nifN* were observed. The fold changes of these genes were high, even 99 times as is expression of *nifH* under cold stress. Similary *anfD, anfH*, and *anfG* were significantly up regulated under cold stress. These genes' expressions decrased after 6h of cold stress compared to 2h, still up regulated compared to the control.

Jouanneau et al. (1985) studied the activity of nitogenase under light intensity of 1200-7000 lux. They showed that when the light intensity increased, the nitrogenase activity was doubled within the first two hours. And under high illumination, 40% of the cytoplasmic proteins were nitrogenase enzyme. In the present study, hydrogen production conditions were set and stress was given to the culture during active hydrogen production phase. One possible reason to the increased expression of genes related with nitrogen metabolism could be the constant illumination of the cultures when shifted to cold stress conditions. The bacteria continuously acquired light energy and might have increased the nitrogenase to channel the light energy to hydrogen.

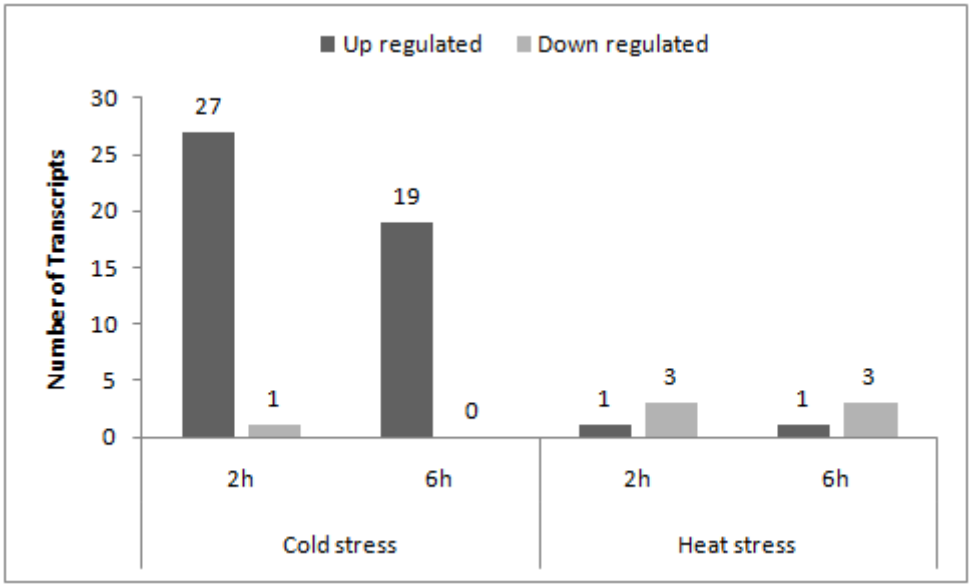


Figure 3. 17 Number of transcripts up and down regulated in nitrogen metabolism under cold and heat stress

Table 3. 4 Changes of some important genes of nitrogen metabolism under cold and heat stress

Gene	Function	Cold Stress		Heat Stress	
		Reg.	Fold change	Reg.	Fold Change
nifB	nitrogenase cofactor biosynthesis protein	Up	6.317	-	-
nifD	nitrogenase molybdenum-iron protein alpha chain	Up	59.112	-	-
nifH	nitrogenase iron protein	Up	99.953	-	-
nifK	nitrogenase molybdenum-iron protein beta chain	Up	29.376	-	-

Table 3.4 (continued)

nifN	nitrogenase molybdenum-iron cofactor biosynthesis protein	Up	6.598	-	-
anfD	nitrogenase iron-iron protein, alpha subunit	Up	23.486	-	-
anfH	nitrogenase iron protein	Up	33.189	-	-
anfG	nitrogenase iron-iron protein, delta subunit	Up	37.180	-	-
glnA	glutamine synthetase	Up	2.249	Down	5.476
glnB	nitrogen regulatory protein P-II	Up	2.756	Down	2.532

The nitrogen availability is sensed by glutamine in the cell. The *glnB* gene, which is involved in the transcriptional regulation of *nifA* gene, which in turn activate nitrogenase genes, was 2.7 fold up regulated under cold stress, while 2.5 fold down regulated under heat stress. The protein GlnB is involved in glutamine synthesis which is required for growth together with the glutamine synthetase encoded by *glnA* gene (Synder and Champness, 2007), down regulation of these gene may contribute the unability to grow under heat stress. These two genes were up regulated under cold stress.

3.7.2 Effect of temperature stress on photosynthesis metabolism of *R. capsulatus*

The Figure 3.18 shows that photosynthesis under heat stress was affected more than cold stress, and this effect was usually in a down regulation manner. Some important genes ans their regulations are shown in Table 3.5. Under heat stress *puf L* and *puf M* together with the *pucA* and *pucC* genes coding for light harvesting II apoproteins, and light harvesting I apoproteins and reaction center, respectively (Bowman et al.,

1999), were down regulated, thus it is meaningful to see decrease in photosynthetic activity under heat stress. The TCA cycle genes such as subunits of succinate dehydrogenase and isocitrate dehydrogenase were also down regulated under heat stress.

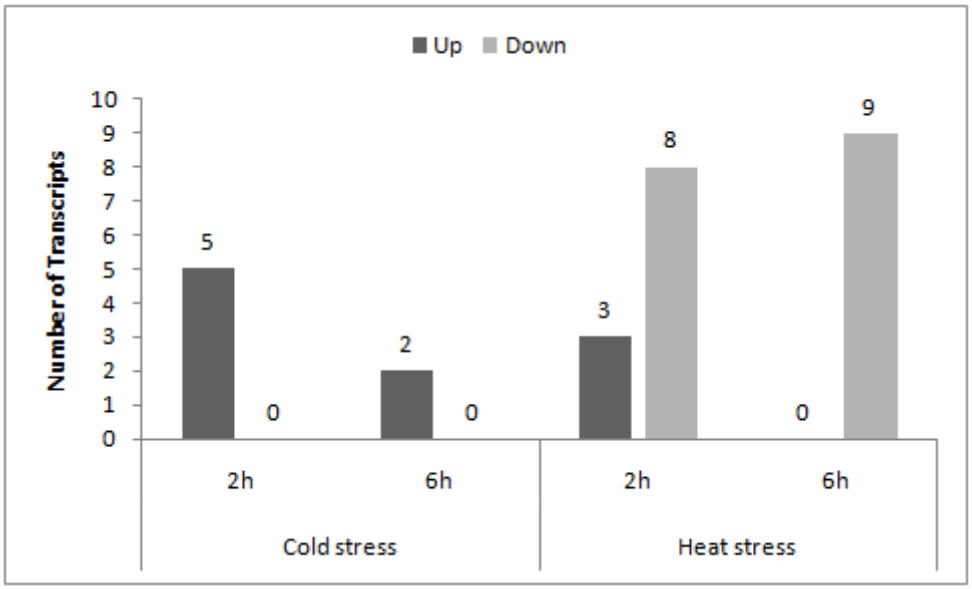


Figure 3. 18 Number of transcripts up and down regulated in photosynthesis metabolism under cold and heat stress

Table 3. 5 Changes of some important genes of photosynthesis under cold and heat stress

Gene	Function	Cold Stress		Heat Stress	
		Reg.	Fold change	Reg.	Fold Change
atpF	ATP synthase F0, B subunit	Up	3.575	-	-
atpC	ATP synthase F1, epsilon subunit	-	-	Down	2.559
atpD	ATP synthase F1, beta subunit	-	-	Down	6.198
pufL	photosynthetic reaction center, L subunit	-	-	Down	3.864

Table 3.5 (continued)

pufM	photosynthetic reaction center, M subunit	-	-	Down	3.102
pucA	light-harvesting protein B-800/850, alpha chain	-	-	Down	2.053
pucC	PucC protein	-	-	Down	2.221
sdhA	succinate dehydrogenase, flavoprotein subunit	-	-	Down	2.147
sdhB	succinate dehydrogenase, iron-sulfur subunit	-	-	Down	3.508
fba	fructose-bisphosphate aldolase	Up	2.056	-	-
fbp	fructose-bisphosphatase	Up	2.748	-	-

The *atpC* and *atpD* genes encoding ATP synthase F1 subunits were other genes down regulated under heat stress.These showed that the energy production mechanisms in the cell were down regulated and necessary energy could not be produced, which might play role in the inability of *R.capsulatus* to grow under heat stress. On the other hand *atpF* coding for ATP synthase F0 B sununit was up regulated under cold stress. The genes for the enzymes fructose bisphosphatase and fructose bisphosphatase aldolase functioning in gluconeogenesis were found to be up regulated together with malate dehydrogenase, an enzyme of TCA cycle.

3.7.3 Effect of temperature stress on electron transport metabolism of *R. capsulatus*

The number of transcripts of electron transport metabolism under cold stress decrased from 2 hours to 6 hours similar to other metabolisms. Under heat stress, the expressions were mostly down regulated (Figure 3.19). Some genes of electron transport metabolism are given in Table 3.6 together with their regulations under cold and stress.

An outstanding result is the high up regulation of ferrodoxin genes under cold stress and down regulation of ferrodoxin V gene under heat stress. Electron transport complex proteins which are encoded by *rnf* genes were highly up regulated under cold stress. Ferrodoxin and flovodoxins are two types of electron carrier proteins which serve as electron donors to nitrogenase. The Rnf proteins also serve as electron donor to nitrogenase under photosynthetic conditions. These proteins thus link electron transport to nitrogen fixation (Klipp et al., 2004). Therefore, the expressions of electron transport genes and nitrogenase genes are in accordance with each other, which is also a result of this study.

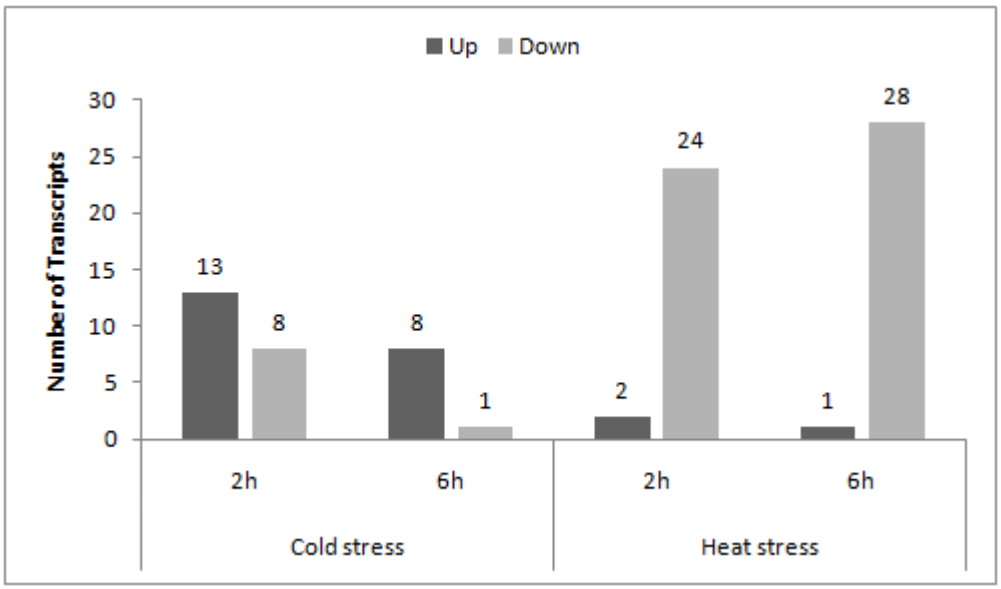


Figure 3.19 Number of transcripts up and down regulated in electron transport metabolism under cold and heat stress

Table 3. 6 Changes of some important genes of electron transport metabolism under cold and heat stress

Gene	Function	Cold Stress		Heat Stress	
		Reg.	Fold change	Reg.	Fold Change
hupA	hydrogenase, small subunit	-	-	Down	2.436
hupB	hydrogenase, large subunit	Up	3.407	Down	3.744
nuoA, B,C,DE,G,H,	NADH-quinone oxidoreductase	-	-	Down	2.118-3.101
fdxN	ferredoxin I	Up	13.582	-	-
fdxC	ferredoxin IV	Up	23.668	-	-
rnfA,B,CD,E,G	electron transport complex protein	Up	7.218-20.417	-	-

Under heat stress the electron transfer flavoproteins α and β were down regulated. Hydogenase genes (*hupA*, *hupB* and *hupC*) were all down regulated under heat stress, while *hupB* gene was up regulated under cold stress. Further, the down regulation of cytochrome c oxidase and NADH quinone oxidoreductase genes (*nuoA*, *nuoB*, *nuoC*, *nuoD*, *nuoE*, *nuoG*, *nuoH*), which link the electron from NADH to ubiquinone forming a protein gradient for ATP sysnthesis (Leif et al., 1995), show the energy impairment under heat stress. This resulted in reduction in growth and survival of the bacteria.

Besides the above gene expressions, thioredoxin was up regulated under heat stress while down regulated under cold stress. This may be because of the function of thioredoxin in redox reductions. Since the other genes for electron transfer were down regulated under heat stress, cells might increase expression of this gene after 2 hours of heat stress to dispose the electrons.

3.7.4 Effect of temperature stress on protein metabolism of *R.capsulatus*

Protein metabolism genes after 2 hours of cold stress were mostly down regulated, as seen in Figure 3.20. Among these down regulated genes, there were genes for proteases, 30S and 50S ribosomal proteins, chaperons including GroS and GroL, and DnaJ and DnaK chaperonins (Table 3.7). The chaperones are among the heat shock proteins and they function in protein folding, repair and degradation. DnaK and DnaJ proteins are chaperones, and also participate in the light harvesting complex I in *R.capsulatus*. It was found by Nickel et al. (1997) that oxygen and light influence the expression levels of *dnaJ* and *dnaK*, so they concluded that these proteins may be necessary for folding and assembly of components of respiratory chain and photosynthetic apparatus in *R.capsulatus*. These two proteins were down regulated by 4.5-5.4 fold after 2 hours of cold stress, then increased to levels of control conditions. Another chaperone ClpB was also down regulated by 12 fold after 2 hours of cold stress. Small heat shock protein gene *ibpA* was also highly (13 fold) down regulated at the same conditions, as do the chaperonins GroS and GroL which were down regulated 6-8 folds.

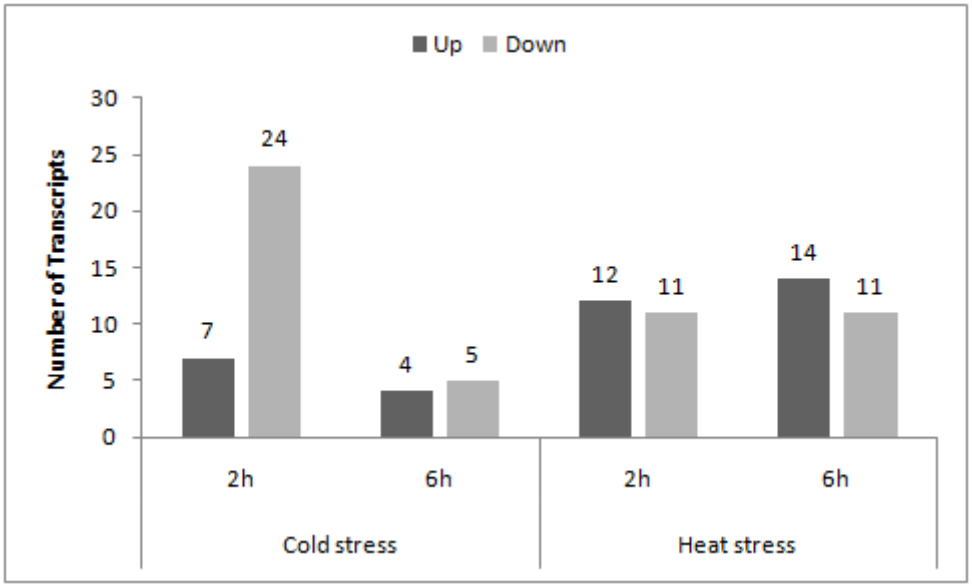


Figure 3. 20 Number of transcripts up and down regulated in protein metabolism under cold and heat stress

Table 3. 7 Changes of some important genes of protein metabolism under cold and heat stress

Gene	Function	Cold Stress		Heat Stress	
		Reg.	**Fold change**	**Reg.**	**Fold Change**
	50S ribosomal proteins	Up	4.036	Up	3.899-8.431
	30S ribosomal proteins	Up	5.586	Up	12.697
clpS	ATP-dependent Clp protease adaptor protein ClpS	Down	3.602	-	-
groS	chaperonin GroS	Down	6.357	-	-
groL	chaperonin GroL	Down	8.799	-	-
clpP	ATP-dependent Clp protease, ATP-binding subunit ClpX	Down	2.995	-	-
ibpA	small heat shock protein IbpA	Down	13.262	-	-
clpA	ATP-dependent Clp protease, ATP-binding subunit ClpA	Down	3.889	-	-
clpB	chaperone ClpB	-	-	Down	2.580
clpP	ATP-dependent Clp protease, ATP-binding subunit ClpX	-	-	Down	3.859
lon	ATP-dependent protease La	Down	5.656	Down	4.331
	Peptidases	Down	2.960-2.430	Down	2.238-3.963

ATP dependent protease genes *hslU*, *hslV*, *clpA*, *clpP*, *clpS*, *lon*, peptidase gene, and *hflk* and *hflC* genes were all down regulated. Both HflK and HflC are associated with the inner membrane having protease activities (Bandyopadhyay et al., 2010). Similar to cold stress, under heat stress, peptidase gene, ATP dependent protease genes *clpS*, *clpX*, *clpP* and *lon* were down regulated. Furthermore, the chaperone *clpB* gene and chaperonin HslO were found to be down regulated. These all show that the protein degradation machineries were down regulated, most probably to protect the already present proteins from degradation.

On the contrary the genes for 30S ribosomal protein and 50S ribosomal protein were found to be up regulated under both cold and heat stress. It is known that transctiption rate decreases with low temperature so with the tendation to incerase transcription, the ribosomal proteins might have been transcribed more. Similarly, since heat stress may degrade proteins, synthesis of the necessary proteins can be increased by increasing the ribosomal protein concentration in the cell.

3.7.5 Effect of temperature stress on cell envelope metabolism of *R. capsulatus*

Most of the cell envelope related genes were down regulated after 2 hours of cold stress, and after 6 hours of cold stress, the number of significantly down regulated genes dropped from 28 genes to only 4 genes. In a similar manner, the number of significantly changed transcripts was dropped from 22 to 12 after 6 hours of heat stress (Figure 3.21). Among these transcripts, 15 were putative membrane protein and 3 had unknown function under heat stress and 12 were putative membrane protein and 9 had unknown function under cold stress. Some important genes and their regulations under cold and heat stress are shown in Table 3.8.

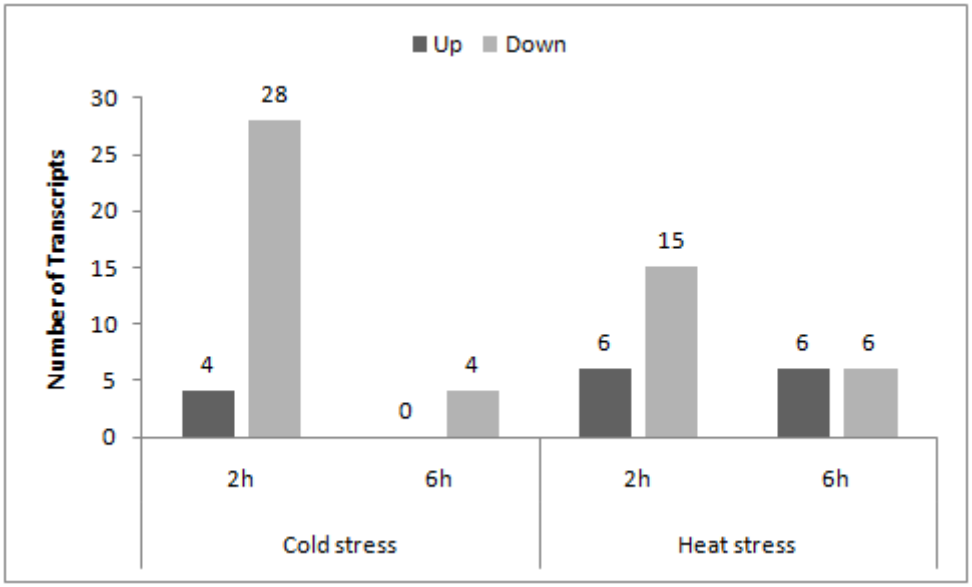


Figure 3. 21 Number of transcripts up and down regulated in cell envelope metabolism under cold and heat stress

Table 3. 8 Changes of some important genes of cell envelope metabolism under cold and heat stress

Gene	Function	Cold Stress		Heat Stress	
		Reg.	Fold change	Reg.	Fold Change
mdoH, lpxD, exoD	Biosynthesis of surface polysaccharides and lipopolysaccharides	Down	4.527, 2.652, 2.716	-	-
ftsI,	Biosynthesis of murein sacculus and peptidoglycan ,	-	-	Down	2.279, 2.222

The gene for rod shape determining protein, *mreC*, was down regulated 2.7 fold after 2 hours of cold stress but showed no significant change after 6 hours of cold stress. This may show the effect of cold stress on the membrane of *R.capsulatus*, since MreC protein has a role in peptidoglycan formation. Returning the expression level to the normal level shows the adaptation to the cold conditions thus the bacteria

could reproduce and increase in number. Similarly, the expression of glucans biosynthesis glucosyltransferase H gene (*mdoH*) which takes place in the biosynthesis of cell wall (Lovering et al., 2007), was significantly down regulated (4.5 fold) after 2 hours of cold stress but it returned to the level of control after 6 hours. Other genes for biosynthesis of surface polysaccharides and lipopolysaccharides such as lipid A biosynthesis acytransferase show similar expression patterns under cold stress. The expression of the gene *pncA* encoding pyrazinamidase/nicotinamidase showed a similar pattern under cold stress, however it was up regulated 3.5 fold after 6 hours of heat stress, since the expression of this gene is induced in response to stress like heat shock (Silva et al., 2009). On the other hand, under heat stress the enzyme peptidoglycan synhtetase which has role in peptidoglycan-based cell wall biogenesis showed down regulation with similar fold changes both in 2 hours and 6 hours. This may mean that the damage of cold stress on the membrane can be reversed upon time passing while it cannot be reversed with heat stress.

3.7.6 Effect of temperature stress on lipid metabolism of *R. capsulatus*

Fatty acid structure is highly sensitive to temperature. Fatty acids mostly occur in the cell membrane structure as the acyl constituents of phospholipids in bacteria. Thus, the effect of temperature stress on the lipid metabolism of bacteria may be parallel to that on cell membrane. Maintining the cell membrane composition has a vital importance for the growth and survival of the bacteria when exposed to low temperatures, since transport of substrates are effected with the membrane.

Under heat stress condition the number of up regulated genes related to lipid metabolism are higher after 2 hours and their fold decreased after 6 hours (Figure 3.22). Under cold stress, after 6 hours the expressions of lipid metabolism genes turned to the levels of control condition.

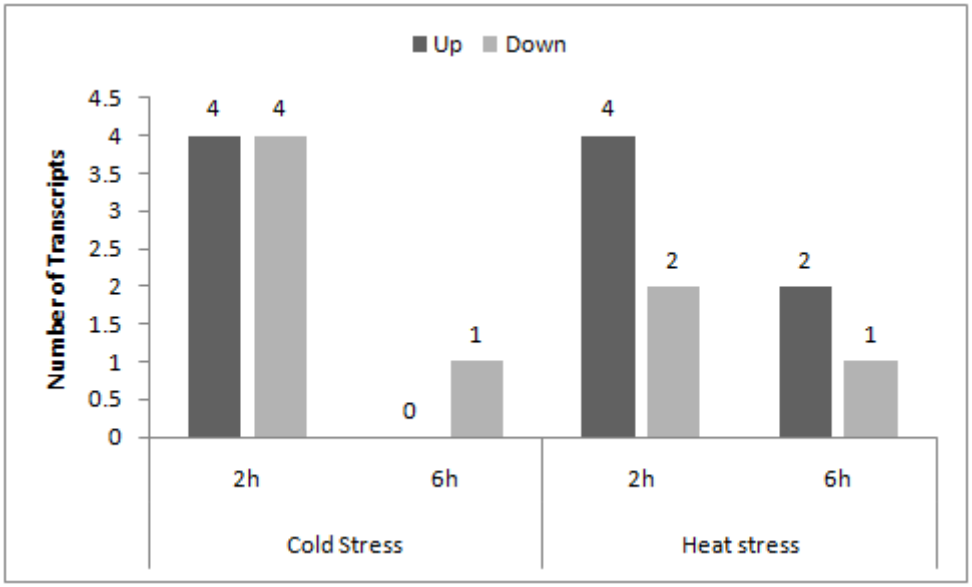


Figure 3. 22 Number of transcripts up and down regulated in lipid metabolism under cold and heat stress

Table 3. 9 Changes of some important genes of lipid metabolism under cold and heat stress

Gene	Function	Cold Stress		Heat Stress	
		Reg.	Fold change	Reg.	Fold Change
acpP	acyl carrier protein	Up	3.545	Up	4.132
pmtA, idi	Biosynthesis of fatty acids and phospholipids	-	-	Up	3.117, 4.085
phbB	acetoacetyl-CoA reductase	Up	2.664	-	-

The acyl carrier protein (Table 3.9) is a very important protein in the fatty acid biosynthesis since it carries the acyl intermediates and interacts with all the enzymes of fatty acid biosynthesis, except acetyl-CoA carboxylase. This gene was up regulated after 2 hours of both cold and heat stress. This may show the tendation to increase fatty acd synthesis, since temperature stress gives harm to the membrane. Acetyl-CoA carboxylase catalyzes the first step in fatty acid biosynthesis, and the

gene for acetyl-CoA carboxylase carboxyltransferase α and β were down regulated under cold stress.

Phosphatidylcholine is a major phospholipid of many plasma membranes. Phosphatidylethanolamine N-methyltransferase is an enzyme functioning in the synthesis of this membrane phospholipid (Furtado et al., 2002), and the gene for this enzyme was found to be up regulated under heat stress, showing that the bacteria was trying to synthesize the membrane components, which might have been damaged upon heat shock. This idea can be supported by another up regulated gene: isopentenyl disphosphate delta isomerase. This enzyme is involved in mevalonate pathway of production of isoprenoids, which can be attached to some proteins to make them incorporate into the membrane, thus they play role in membrane maintenance.

The enzyme propionate-CoA ligase takes place in the utilization of the fatty acid propionate via TCA cycle. The gene for this enzyme was up regulated under cold stress. In *R.capsulatus*, volatile fatty acids such as acetic acid and butric acid can be stored in the form of polyhydroxybutryic acid. Acetoacetyl-CoA reductase function in the synthesis of this storage polymers. The gene for this enzyme was found to be up regulated after 2 hours of cold stress.

3.7.7 Effect of temperature stress on nucleic acid metabolism of *R. capsulatus*

The number of genes of nucleic acid metabolism having significant expression changes decrased from 2 hours to 6 hours of cold stress application (Figure 3.23). Some important genes of nucleic acid metabolism with their function and regulations under cold and heat stress are shown in Table 3.10.

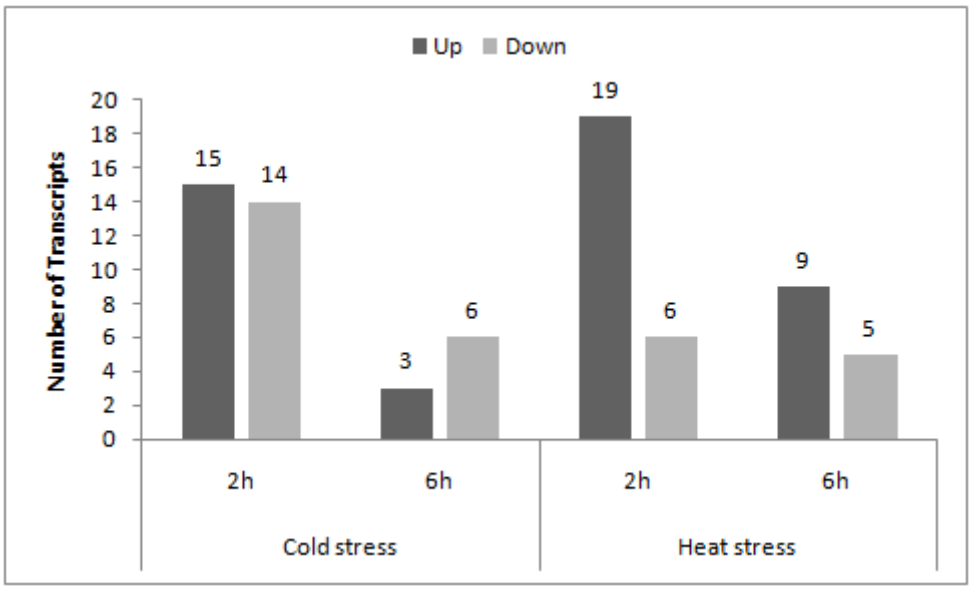


Figure 3. 23 Number of transcripts up and down regulated in nucleic acid metabolism under cold and heat stress

Genes responsible for DNA replication, recombination, transcription regulators and transcription factors are among the significantly down or up regulated genes. For instance DNA primase and DNA polymerase genes were down regulated 2 fold after 2 hours of cold stress application, then they returned back to the basal level, so that the cells continued to replicate.

Ribonuclease E which is an endonuclease without any sequence specificity degrades mRNA which is required for the regulation of levels of specific mRNAs in the cell upon environmental changes (Mackie, 1998). The up regulation of ribonuclease E under cold stress is thus convenient. Similarly the RNA chaperone Hfq was up regulated at this condition. This protein is an RNA binding protein having regulatory function on gene expression, growth of bacteria and stress tolerance (Chambers and Bender, 2011). Another gene encoding polyribonucleotidyltransfrease which has role in degredation of RNA molecules was up regulated 6 fold, this expressional change is convenient with that of ribonuclease E and RNA chaperone Hfq. These results may show the quick transcriptional regulation taking place in *R.capsulatus* under cold stress, but their expression were in a trend similar to the control condition after

6 hours of cold stress. This again shows the adaptation of these bacteria to cold stress.

Table 3. 10 Changes of some important genes of nucleic acid metabolism under cold and heat stress

Gene	Function	Cold Stress		Heat Stress	
		Reg.	Fold change	Reg.	Fold Change
mopA	molybdenum transport operon repressor MopA	up	2.673	-	-
rpoN	RNA polymerase sigma-54 factor	up	6.031	-	-
hfq	RNA chaperone Hfq	up	2.452	-	-
rne	ribonuclease E	up	3.679	-	-
pnp	Degradation of RNA	up	6.457	-	-
nusG	transcription antitermination protein	-	-	up	3.113
rnp	ribonuclease P	-	-	up	2.065
rpoH	RNA polymerase sigma-32 factor	-	-	down	3.361
rpoD	RNA polymerase sigma factor	-	-	down	2.919

The alternative sigma factor RpoN, also called sigma 54 factor, was up regulated 6 fold under cold stress. This protein recognizes the *nifA* genes which are activators of nitrogenase and other *nif* genes (Foster-Hartnett and Kranz, 1994). Up regulation of RpoN is then relevant to the high up regulation of nitrogenase genes.

The molbindin MopA gene was up regulated after 2 hours of cold stress. This protein represses the expression of *anfA* gene encoding transcriptional activator of Fe-nitrogenase, and the *modABC* genes coding for molybdenum transporters (Wiethaus et al., 2006). *modB* was up regulated under cold stress in response to the increase of nitrogenase genes.

The gene for transcriptional terminator protein NusG is one of the genes having significant expressional change under heat stress. It is a transcription factor affecting the rate of RNA polymerase synthesizing the RNA molecules. Burova et al. (1995) demonstrated that NusG accelerates transcription elongation rate. This gene was up regulated under heat stress, implying that the cells tried to cope with the cellular damage by increasing the transcription of some proteins. One another gene whose expression was up regulated was for ribonuclease P, which has a role in maturation of tRNAs. This up regulation seems complementary to up regulation of NusG, since increasing transcription of some genes would increase their translation, so more tRNAs are of need.

In a study conducted by Emetz and Klug (1998) when the temperature was shifted from 32 °C to 40 °C, the expression of *rpoH* gene encoding RNA polymerase sigma 32 factor, was shown to increase 12 fold after 10 minutes, and then started to decrase, so that the expression was 8 fold more in heat stress after 30 minutes. This revealed that *rpoH* of *R.capsulatus* is regulated by heat shock and this gene is necessary of growth. In the present study, under heat stress, this gene was found to be down regulated, together with *rpoD* gene, which encodes the principle sigma factor of *R.capsulatus* and which is essential for cell survival (Pasternak et al., 1996).

The gene *uvrB* encoding the proein UvrB, a component of the UvrABC which is involved in the nucleotide excision repair of DNA, was found to be down regulated by 4.5-6 fold in both heat and cold stress. Besides the repair function in response to damage by UV radiation, UvrB is suggested to have an additional function as DNA polymerase in the cell (Truglio et al., 2006). Taking into consideration of this DNA polymerase activity of UvrB, down regulation might be plausible since DNA

polymerase and primase was also down regulated. DNA damages can be repaired by *recA* gene product. Indeed, it was shown by Henestrosa and coworkers (1997) that upon DNA damage the expression of *recA* gene was stimulated in *R.capsulatus*. Since the heat shock is known to give harm to DNA, up regulation of *recA* gene under heat stress in *R.capsulatus* in this study is meaningful.

Site specific DNA methytransferase gene was up regulated after 2 hours of both heat and cold stress. DNA methylation in prokaryotes protects DNA from degredation by endonucleases. In order to protect the DNA to which temperature stress gives harm, up regulation of this gene may be a good way.

3.7.8 Effect of temperature stress on transporters and binding proteins of *R. capsulatus*

Heat stress affected the transporters and binding proteins of *R.capsulatus* more than cold stress (Figure 3.24). Most of the down regulated genes belong to the transporters of amino acids and peptides, and they are called polyamine ABC transporters, as shown in Table 3.11. Besides the polyamine ABC transporters, genes of spermidine/putrescine ABC trasnporters (*potD, potF, potG, potH*) had significant and outstanding down regulation under heat stress. Spermidine and putrescine are polyamines which are required for growth since they stimulate RNA and protein synthesis (Raina and Jänne, 1975). Furthermore, having high affinity for nucleic acids, they stabilize the secondary structures of DNA and RNA. They also stabilize the cell membrane (Tabor and Tabor, 1976). These results may give an idea of why *R.capsulatus* cells could not grow under heat stress.

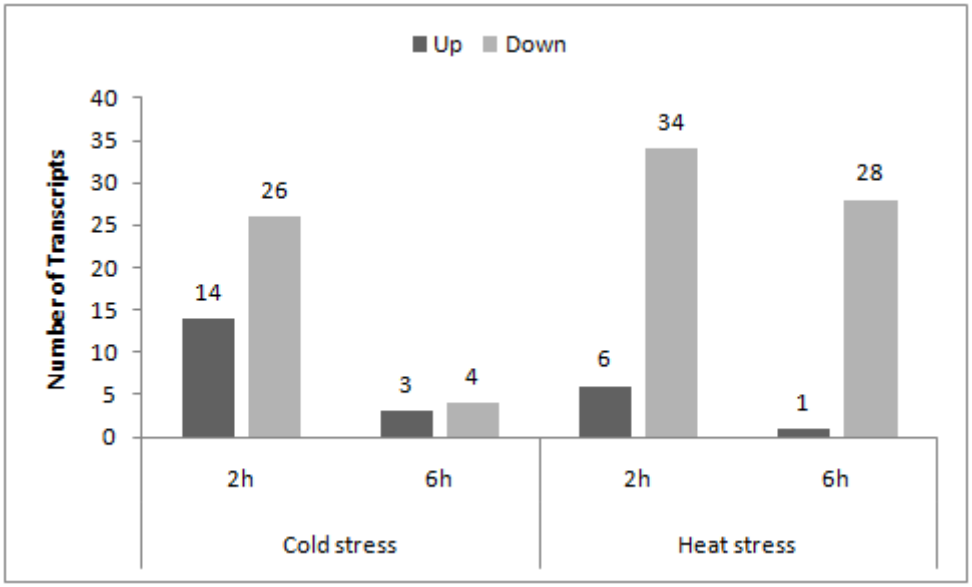


Figure 3. 24 Number of transcripts up and down regulated in transporter and binding proteins metabolism under cold and heat stress

Table 3. 11 Changes of some important genes of transporters and binding proteins under cold and heat stress

Gene	Function	Cold Stress		Heat Stress	
		Reg.	Fold change	Reg.	Fold Change
modB	molybdenum ABC transporter, permease protein ModB	up	3.113	-	-
potA,B,D,F,G,H,I	spermidine/putrescine ABC transporter, periplasmic spermidine/putrescine-binding protein	-	-	down	2.010-14.108
dctP	Trasnsporter for organic acids, carbohydrates, alcohols	-	-	down	10.165

Cations and iron carrying compounds, and transporters of nucleosides showed also down regulation under heat stress. Moreover, the gene for iron siderophore had a significant up regulation under heat stress (7.6 fold). Iron is almost always an essential mineal for bacteria, and sideophores take the iron from the environment and

make it available for the organism, because iron is not always available for the bacterium (Neilands, 1995). Genes for transporters for carbohydrate and organic acids were down regulated and this is convenient with the physiological results since HPLC results for acetate showed that approximately 5mM acetate were remained unutilized in the medium after heat stress. Some transporters' substrates could not be defined yet.

The transporter and binding proteins genes whose expressions were significantly changed under cold stress were that of aminoacids and peptide transporters, some of which were up regulated, some were down regulated. Transporters for cations and iron carrying compounds, such as iron (III) transporter was down regulated. Iron sideophore gene was up regulated under cold stress too, but only 2 folds, that is less when compared to heat stress. Molybdenum ABC transporter encoded by *modB*, was up regulated after 2 hours of cold stress. Since molybdenum is a cofactor for nitrogenase gene and since nitrogenase genes were up regulated under this condition, up regulation of *modB* gene was an expected result, because increasing nitrogenase gene expression means increasing molybdenum need. Some transporters still have undefined substrates.

3.7.9 Effect of temperature stress on biosynthesis of cofactors, prosthetic groups and carriers of *R. capsulatus*

As it is seen from the Figure 3.25, after 6 hours of cold stress, the expressions of the genes for biosynthesis of cofactors, prosthetic groups and carriers returned to the levels of normal condition.

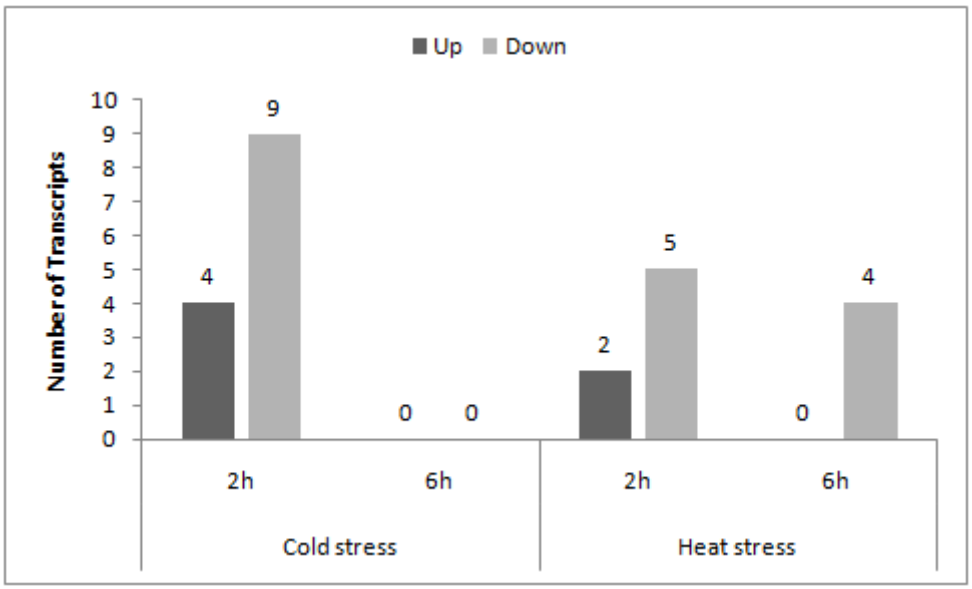


Figure 3. 25 Number of transcripts in biosynthesis of cofactors, prosthetic groups and carriers under cold and heat stress

The enzymes taking place in the synthesis of ubiquinone and menaquinone were down regulated under cold stress. Ubiquinone and menaquinone are lipid solube and they are components of electron transport chain placed on the membrane. In most of the bacteria, reducing equivalents are transferred to a common pool of ubiquinones and menaquinones which are then reoxidized by a final electron acceptor. Menaquinone also takes place in the synthesis of pyrimidines. It was found that under anaerobic conditions ubiquinone level decreased 10 fold in *E.coli* (Soballe and Poole, 1999). In *R.capsulatus*, the presence of an alternative ubiquinone-cytochrome c oxidoreductase electron transport which has a low enzyme activity was found. The presence of this enzyme system in the photosynthetic electron transport was attributed to the prevention of attenuation of quinone cycle which might occur when the quinone pool was highly reduced causing the turnover of cytochrome bc_1 complex. Thus this ubiquinol cytochrome c oxidoreductase complex constitutes an alternative to cytochrome bc1 complex under photosynthetic and anaerobic respiration (McEwan, 1994).

Genes for the biosynthesis of folic acid were down regulated under both cold and heat stress. Folic acid is one of the B vitamins and it is a cofactor for various reactions in the cells since it has an ability to accept or donate one-carbon units (Duthie, 1999). It is also required for growth and it takes role in nucleic acids metabolism being a coenzyme in thymine synthesis (Stokes, 1944). Decrease in the biosynthesis of folic acid may decrease growth and repairing the damage caused by temperature stress both under cold and heat stress.

Under heat stress, genes for enzymes of pantothenate and coenzyme A showed down regulation. They function as acyl carriers, therefore thay play important roles in the fatty acid metabolism (Begley et al., 2001). Toennies et al. (1966) found that pantothenate is an essential growth factor for *S. faecalis*. They also cocluded that synthesis of lipid being the membrane building blocks involve acyl transport and acyl transport is dependent on pantothenate in the form of coenzyme A.

An important change occurred in the *sufC* and *sufD* genes under heat stress in that they were significantly down regulated by 4-6 folds (Tables 3.12). These genes are two of the genes for Fe/S proteins which participate in the light mediated electron transport and stress-induced regulation of genes in photosynthetic organisms. Furthermore, Fe/S proteins function to serve as a brigde between two proteins, an outstanding example is being the brigde between nitrogenase and photosystem I. SufC protein function as an ATPase while SufD protein take place in the assimilation of iron (Shen and Golbeck, 2006). The down regulation of these two genes are convenient with the genes of photosynthesis and electron transport genes which were also down regulated under heat stress.

There are genes for cofactors whose role could not be determined yet.

CHAPTER 4

CONCLUSIONS

In the present study, the effects of heat and cold stress on hydrogen production and other metabolisms of *Rhodobacter capsulatus* DSM1710 were investigated using both physiologic and genomics approaches. The control condition of the stress applications was selected as 30 °C, and cold stress (4 °C) and heat stress (42 °C) were compared with the control. Hydrogen production, pH of the medium, bacterial growth and acetate consumptions of stress conditions were compared to the control. Moreover, by microarray experiments with the custom design Affymetrix GeneChip®s and analysis of microarray results, the effects of temperature stress on different metabolisms with respect to the control were studied. The genes having expressional changes of ≥ 2 fold together with statistical analysis ($p \leq 0.1$) compared to the expressions of the control condition were taken into account in the microarray analyses.

Based on the obtained results and discussions of these results, the followings were deduced:

- Upon heat stress, the bacterial growth stopped, however, under cold stress bacteria continued to grow at a slower rate. Hydrogen production was ceased in both stress conditions.

- Acetate was utilized totally within four days under control, and six days under cold stress. However, under heat stress some acetate remained in the medium even after seven days.

- Isolation of total RNA of *R.capsulatus* was optimized and high concentrations of RNA with high purity required for microarray experiments were successfully isolated.

- Whole genome microarray chip was designed for *R.capsulatus*, and was manufactured by Affymetrix. Hybridization of the labeled cDNA onto this custom design GeneChip® for *R.capsulatus* was also successful showing that the microarray chips worked well for this bacterium.

- According to the gene lists from the fold change (≥ 2.0) and significance analysis ($p \leq 0.1$), genes were categorized into appropriate metabolism according to their roles defined by the research group that sequenced the whole genome sequence of *R.capsulatus*.

- Effects of cold and heat stress on each metabolism of *R.capsulatus* were investigated according to the changes in the expressions of the genes in the corresponding metabolism. The results revealed that the nitrogen, photosynthesis and electron transport metabolisms, which carry vital importance for hydrogen production, were highly affected from heat stress showing why hydrogen production was stopped under heat stress.

- Cell envelope and lipid metabolisms were also affected by temperature stress, as expected. Gene expressions changed in order to keep the membrane intact and stable. In parallel to the effects on membrane, the transporters and binding proteins were affected by the temperature stress. Moreover, according to the needs of the up regulated gene products, besides the transporters and binding proteins, biosynthesis of cofactors, carrier proteins and prosthetic groups were up or down regulated.

- Proteins and nucleic acids were affected by teamperature stress. In order to protect the already translated proteins in the cell, protein degredation machineries were down regulated, while cells tended to increase the translation.

- The results obtained from this study can be used to make genetic modifications on *R.capsulatus* and other PNS bacteria in order to attain better hydrogen producing bacteria which can be employed in large scale photobioreactors.

- Global effects of heat stress on a heat adapted strain of *Rhodobacter capsulatus* and *hup*$^-$ strain of *R.capsulatus* will be investigated by microarray chip designed in this study microarray analysis

- Mixed effect of heat and high light intensity, which are two factors limiting hydrogen production in outdoor conditions, will also be investigated by microarray analysis.

- Gathering the data from the investigations mentioned above may lead more efficient genetic modifications on *Rhodobacter capsulatus* and other species of PNS bacteria. Therefore, continuous hydrogen production without the negative effect of heat and cold stress can be achieved in the future.

REFERENCES

Affymetrix, Sample pooling for microarray analysis: A statistical assessment of risks and biases, Technical note, no. 701494 Rev. 2, 2004.

Afsar, N., Özgür, E., Gürgan, M., Akköse, S., Yücel, M., Gündüz, U., Eroğlu, İ. (2011). Hydrogen productivity of photosynthetic bacteria on dark fermenter effluent of potato steam peels hydrolysate. *International Journal of Hydrogen Energy, 36*, 432-438.

Akkerman, I., Jannsen, M., Rocha, J., Wijffels, R.H. (2002). Photobiological hydrogen production: photochemical efficiency and bioreactor design. *International Journal of Hydrogen Energy, 27*, 1195–1208.

Akköse, S., Gündüz, U., Yücel, M., Eroğlu, İ. (2009). Effects of ammonium ion, acetate and aerobic conditions on hydrogen production and expression levels of nitrogenase genes in *Rhodobacter sphaeroides* O.U.001. *International Journal of Hydrogen Energy, 24,* 8818-8827.

Androga, D.D., Özgür, E., Gündüz, U., Yücel, M., Eroğlu, İ. (2011). Factors affecting the longterm stability of biomass and hydrogen productivity in outdoor photofermentation. *International Journal of Hydrogen Energy.* doi:10.1016/j.ijhydene.2010.12.054.

Arsène F., Tomoyasu, T., Bukau, B. (2000). The heat shock response of *Escherichia coli*. *International Journal of Food Microbiology, 55* ,3–9.

Asada, Y., Oshawa, M., Nagai, Y., Ishimi, K., Fukatsu, M., Hideno, A. et al. (2008). Re-evaluation of hydrogen productivity from acetate by some photosynthetic bacteria. *International Journal of Hydrogen Energy, 33*, 5147–5150.

Avcıoğlu, S.G., Androga, D.D., Uyar, B., Özgür, E, Gündüz, U., Yüzel, M., Eroğlu, İ. (2009). Continuous hydrogen production by *R.capsulatus* on acetate in panel photobioreactors. HYPOTHESIS VIII Lisbon (Portugal).

Avcioglu, S.G., Özgür, E., Eroğlu, İ., Yücel, M., Gündüz, U. (2011). Biohydrogen production in an outdoor panel photobioreactor on dark fermentation effluent of molasses. *International Journal of Hydrogen Energy.* doi:10.1016/j.ijhydene.2010.12.04.

Bandyopadhyay, K., Parua, P.K., Datta, A.B., Parrack, P. (2010). *Escherichia coli* HflK and HflC can individually inhibit the HflB (FtsH)-mediated proteolysis of λCII *in vitro*. *Archieves of Biochemistry and Biophysics, 501(2),* 239-243.

Barbosa, M.J., Rocha, J.M.S., Tramper, J., Wijffels, R.H. (2001). Acetate as a carbon source for hydrogen production by photosynthetic bacteria. *Journal of Biotechnology, 85*, 25–33.

Basak, N. & Das, D. (2007). The prospect of purple non-sulfur (PNS) photosynthetic bacteria for hydrogen production: the present state of the art. *World J. Microbiol. Biotechnol., 23*,31–42

Beales, N. (2004). Adaptation of microorganisms to cold temperatures, weak acid preservatives, low pH, and osmotic stress: A review. *Comprehensive Reviews in Food Science and Food Safety, 3*, 1-20.

Begley, T.P., Kinsland, C., E. (2001). The biosynthesis of coenzyme a in bacteria. *Vitamins and Hormones, 61*, 157-171.

Bergey, D.H. & Holt, J.G. (1994). *Bergey's Manual of Determinative Bacteriology* (9th Ed.). Philedelphia: Lippincott Williams & Wilkins

Berry, E.D. & Foegeding, P.M., 1997. Cold temperature adaptation and growth of microorganisms. *J. Food Prot., 60,* 1583–1594.

Biebl, H. & Pfennig, N. (1981). Isolation of member of the family *Rhodosprillaceae*. In: Starr MP, Stolp H., Trüper H. G., Balows A., Schlegel H. G., (Ed.). *The prokaryotes*, (267–273). New York: Springer.

Bowman, W., Du, S., Bauer, C.E., Kranz, R.G. (1999). *In vitro* activation and repression of photosynthesis gene transcription in *Rhodobacter capsulatus. Molecular Microbiology, 33(2), 429-437.*

Briiutigam, E., Fiedler, F., Woitzik, D., Flammann, H.T., Weckesser, J. (1988). Capsule polysaccharide-protein-peptidoglycan complex in the cell envelope of *Rhodobacter capsulatus. Arch Microbiol. 150*, 567- 573.

Burove, E., Hung, S.C., Sagitov, V., Stitt, B.L., Gottesman, E. (1995). *Escherichia coli* NusG protein stimulates transcription elongation rates in vivo and in vitro. *Journal of Bacteriology, 177(5),* 1388-1392.

Burris, R.H. (1991). Nitrogenases. *The Journal of Biologica Achle Misty, 266*, 9339-9342.

Catucci, L., Depalo, N., Lattanzio, V.M.T., Agostiano, A., Corcelli, A. (2004). Neosynthesis of cardiolipin in *Rhodobacter sphaeroides* under osmotic stress. *Biochemistry, 43*, 15066–15072.

Chambers, J.R. & Bender, K.S. (2011). The RNA chaperone Hfq is important for growth and stress tolerance in *Francisella novicida*. *PLoS ONE, 6(5)*, doi:10.1371/journal.pone.0019797.

Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem., 162*, 156-159.

Claassen, P.A.M. & de Vrije, T. (2006). Non-thermal production of pure hydrogen from biomass: HYVOLUTION. *International Journal of Hydrogen Energy , 31,* 1416 – 1423

Colbeau, A., Richaud, P., Toussaint, B, Caballero, F.J., Elster, C., Delphin, C. et al. (1993). Organization of the genes necessary for hydrogenase expression in *Rhodobacter capsulatus.* Sequence analysis and identification of two *hyp* regulatory mutants. *Molecular Microbiology, 8(1),* 15-29.

Conner, D.E. & Kotrola, J.S. (1995). Growth and survival of *Escherichia coli* O157:H7 under acidic conditions. *Appl. Environ. Microbiol., 61(1),* 382-385.

Das, D. & Veziroğlu, T.N. (2001). Hydrogen production by biological processes: a survey of literature. *International Journal of Hydrogen Energy, 26*, 13–28

Das, D. & Veziroğlu, T.N. (2008). Advances in biological hydrogen production processes. *International Journal of Hydrogen Energy, 33*, 6046-6057.

Dharmadi, Y. & Gonzalez, R. (2004). DNA microarrays: Experimental issues, data analysis, and application to bacterial systems. *Biotechnol. Prog., 20,* 1309−1324.

Dumur, CI., Nasim, S., Best, A.M., Archer, K.J., Ladd, A.C., Mas, V.R. et al. (2004). Evaluation of quality-control criteria for microarray gene expression analysis. *Clinical Chemistry , 50(11),* 1994–2002.

Duthie, S.J. (1999). Folic acid deficiency and cancer: mechanisms of DNA instability. *Br Med Bull, 55 (3)*, 578-592.

Ehrenreich, A. (2006). DNA microarray technology for the microbiologist: an overview *Appl Microbiol Biotechnol., 73*, 255–273.

El-Rab, S.M.F.G., Shoreit, A.A.F., Fukumori, Y. (2006). Effects of cadmium stress on growth, morphology, and protein expression in *Rhodobacter capsulatus* B10. *Bioscience, Biotechnology, and Biochemistry, 70(10),* 2394-2402.

Emetz, D & Klug, G. (1998). Cloning and characterizationof the *rpoH* gene of *Rhodobacter capsulatus*. *Mol. Gen. Genet., 260*, 212-217.

Eroğlu, İ., Sevinç, P., Gündüz, U., Yücel, M. (2010). The effect of temperature and light intensity on hydrogen production by *Rhodobacter capsulatus*. Proceedings of World Hydrogen Energy Conference, Germany, 18, 26-31.

Falciani, F. (2007). *Microarray technology through applications*. New York: Taylor & Francis Group.

Farrel, J. & Rose, A. (1967). Temperature effects on microorganism. *Annu. Rev. Microbiol., 21*, 101-120.

Fleige, S. & Pfaffl, M.W. (2006). RNA integrity and the effect on the real-time qRT-PCR performance. *Molecular Aspects of Medicine, 27*,126–139.

Foster-Hartnett, D. & Kranz, R.G. (1994).The *Rhodobacter capsulatus* glnB gene is regulated by NtrC at Ttndem rpoN-independent promoters. *Journal of Bacteriology, 176(16)*, 5171-5176.

Furtado, V.C.S., Takiya, C.M., Braulio, V.B. (2002). Phosphatidylethanolamine *N*-methyltransferase activity is increased in rat intestinal brush-border chronic ethanol ingestion. *Alcohol and Alcoholism, 37(6),* 561-565.

GeneChip® expression analysis technical manual (n.d.). Retrieved January 28, 2011 from http://www.affymetrix.com/support/technical/manuals.affx.

GeneChip® customexpress™ array design guide (n.d.). Retrieved January 28, 2011 from http://media.affymetrix.com/support/technical/other/custom_design_manual.pdf.

Goldstein, A., Goldstein, D.B., Lowney, L.I. (1964). Protein synthesis at 0 °C in *Escherichia coli*. *Journal of Molecular Biology, 9(1*), 213-235.

Gomes, S.L. & Simão, R.C.G. (2009). Stress responses: Heat. *Encyclopedia of Microbiology, 1*, 464-476.

Guez, J.S., Coutte, F., Drucbert, A.S., Chihib, N.E., Danzé, P.M., Jacques, P. (2009). Resistance of the cell wall to degradation is a critical parameter for isolation of high quality RNA from natural isolates of *Bacillus subtilis. Arch Microbiol , 191*, 669–673.

Gupta, R.S. (2010). Molecular signatures for the main phyla of photosynthetic bacteria and their subgroups. *Photosynth. Res*., 104, 357–372.

Hallenbeck, P. & Benemann, J. (2002). Biological hydrogen production; fundamentals and limiting processes. *International Journal of Hydrogen Energy, 27*, 1185–1193.

Hardiman, G. (2004). Microarray platform comparisons and contrasts. *Pharmacogenomics, 5(5),* 487–502.

Haselkorn, R., Lapidus, A., Kogan, Y., Vlcek, C., Paces, J., Paces, V. et al. (2001). The *Rhodobacter capsulatus* genome. *Photosynthesis Research, 70*, 43–52.

He, D., Bultel, Y., Magnin, J.P., Willison, J.C. (2006). Kinetic analysis of photosynthetic growth and photohydrogen production of two strains of *Rhodobacter capsulatus. Enzyme and Microbial Technology, 38,* 253–259.

Heller, M.J. (2002). DNA microarray technology: Devices, systems and applications. *Annu. Rev. Biomed. Eng., 4*, 129–53.

Henestrosa, A.R.F., Labazi, M., Lopez, M.M., Barbe, J. (1997). Functional analysis of the *recA* promoter of *Rhodobacter capsulatus. Mol. Gen. Genet., 255*, 487-494.

Hillmer, P & Gest, H. (1977). H_2 Metabolism in the photosynthetic bacterium *Rhodopseudomonas capsulata*: H_2 production by growing cultures. *Journal of Bacteriology*, 724-731.

Ho, T. & Karri, V. (2010). Basic tuning of hydrogen powered car and artificial intelligent prediction of hydrogen engine characteristics. International Journal of Hydrogen Energy, 35(18), 10004-10012.

Imhoff, J.F., (1995). Taxonomy and physiology of phototropic purple bacteria and gren sulfur bacteria. *Anoxygenic Photosynthetic Bacteria, 2*, 1-15.

Imhoff, J.F., Hiraishi, A., Süling, J. (2005). Anoxygenic Phototrophic Purple Bacteria *Bergey's Manual of Systematic Bacteriology*, 119-132.

Imhoff, J.F. (2006). The Phototrophic Alpha-Proteobacteria. *Prokaryotes, 5*, 41–64.

Jouanneau, Y., Wong, B., Vignais, P.M. (1985). Stimulation by light of nitrogenase synthesis in cells of *Rhodopseudomonas capsulata* growing in N-limited continuous cultures. *Biochimica et Biophysica Acta - Bioenergetics, 808 (1)*,149-155.

Kars, G., Gündüz, U., Yücel, M., Türker, L., Eroğlu, İ. (2006). Hydrogen production and transcriptional analysis of nifD, nifK and hupS genes in *Rhodobacter sphaeroides* O.U.001 grown in media with different concentrations of molybdenum and iron. *International Journal of Hydrogen Energy, 31(11)*, 1536-1544.

Kars, G., Gündüz, U., Rakhely, G., Yücel, M., Eroğlu, I., Kovacs, K.L. (2008). Improved hydrogen production by uptake hydrogenase deficient mutant strain of *Rhodobacter sphaeroides* O.U.001. *International Journal of Hydrogen Energy, 33*, 3056-3060.

Kars, G. & Gündüz,U. (2010). Towards a super H_2 producer: Improvements in photofermentative biohydrogen production by genetic manipulations. *International Journal of Hydrogen Energy, 35*, 6646-6656.

Klipp, W., Masepohl, B., Gallon, J.R., Newton, W.E. (2004). Genetics and regulation of nitrogen fixation in free-living bacteria. Dordrecht, Netherlands: Kluwer Academic Publisher.

Ko R., Smith, L.T., Smith, G.M. (1994). Glycine betaine confers enhanced osmotolerance and cryotolerance on *Listeria monocytogenes. Journal of Bacteriology, 176,* 426-431.

Koku, H.,Eroğlu, İ., Gündüz, U., Yücel, M., Türker, M. (2002). Aspects of the metabolism of hydrogen production by *Rhodobacter sphaeroides. International Journal of Hydrogen Energy, 27,* 1315–1329.

Kotay, S.M. & Das, D. (2008). Biohydrogen as a renewable energy resource-Prospects and potentials. *International Journal of Hydrogen Energy, 33*, 258–263.

Kothari,R., Buddhi, D., Sawhney, R.L. (2008). Comparison of environmental and economic aspects of various hydrogen production methods. *Renewable and Sustainable Energy Reviews, 12*, 553–563.

Larimer, F.W., Chain, P., Hauser, L., Lamerdin, J., Malfatti, S., Do, L. et al. (2003). Complete genome sequence of the metabolically versatile photosynthetic bacterium *Rhodopseudomonas palustris. Nature Biotechnology, 22,* 55 – 6.

Leif, H., Sled, V.D., Ohnishi, T., Weiss, H., Friedrich, T. (1995). Isolation and characterization of the proton-translocating NADH : ubiquinone oxidoreductase from *Escherichia coli. Eur. J. Biochem.,230*, 538-548.

Levin, D.B., Pitt, L., Love, M. (2004). Biohydrogen production: prospects and limitations to practical application. *International Journal of Hydrogen Energy, 29*, 173–185.

Lovering, A.L.,de Castro, L.H., Lim, D., Strynadka, N.C.J. (2007). Structural insight into the transglycosylation step of bacterial cell-wall biosynthesis. Science, 315 (5817), 1402-1405.

Macario, A .J. L., & de Macario E.J. (2000). Heat Shock Response, Overview. In: The Encyclopedia of Stress Ed: G. Fink Academic Press, CA. pp. 350-357.

Mackie, G.A. (1998). Ribonuclease E is a 5'-end-dependent endonuclease. *Nature, 395 (6703)*, 720-723.

Mastroleo, F., Houdt, R.V., Leroy, B., Benotmane, M.A., Janssen, A., Mergeay, M. et al. (2009). Experimental design and environmental parameters affect *Rhodospirillum rubrum* S1H response to space flight. *The ISME Journal, 3*, 1402–1419.

Maxwell, I.H., Maxwell, F., Hahn, W.E. (1976). Removal of RNase activity from DNase by affinity chromatography on agarose-coupled aminophenylphosphoryl-uridlne-2′(3′)-phosphate. *Nucleic Acids Research, 4(1)*, 241-246.

McEwan, A.G. (1994). Photosynthetic electron transport and anaerobic metabolism in purple non-sulfur phototrophic bacteria. *Antonie van Leeuwenhoek, 66*, 151-164.

McKinlay, J.B., & Harwood, C.S. (2010). Photobiological production of hydrogen gas as a biofuel. *Current Opinion in Biotechnology, 21*, 244–251.

Mejía, R., Eichelmann**,** M.C.G., Fernández, M.S. (1995). Membrane fluidity of *Escherichia coli* during heat-shock. *Biochimica et Biophysica Acta-Biomembranes,1239 (2)*, 195-200.

Melis, A. (2002). Green alga hydrogen production: progress, challenges and prospects. *International Journal of Hydrogen Energy, 27*, 1217-1228.

Methe, B.A., Nelson, K.E., Deming, J.W., Momen, B., Melamud, E., Zhang, X. et al. (2005). The psyrophilic lifestyle as revealed by the genome sequence of *Colwellia psycherythraea* 34H through genomic and proteomic analyses. Proceedings of the National Academy of Sciences (USA), 102, 10913-10918.

Mihoub, F., Mistou, M.Y., Guillot, A., Leveau, J.Y., Boubetra, A., Billaux, F. (2003). Cold adaptation of *Escherichia coli*: microbiological and proteomic approaches. *International Journal of Food Microbiology, 89*, 171– 184.

Nakahigashi, K., Yanagi, H., Yura, T. (2001). DnaK chaperone-mediated control of activity of a sigma (32) homolog (RpoH) plays a major role in the heat shock response of *Agrobacterium tumafaciens. J. Bacteriol., 183*, 5302-5310.

Nanqi, R., Wanqian,G., Bingfeng, L., Guangli, C., Jie, D. (2011). Biological hydrogen production by dark fermentation: challenges and prospects towards scaled-up production. *Opinion in Biotechnology, 22*, 365–370.

Nath, K. & Das, D. (2004) Improvement of fermentative hydrogen production: various approaches. *Appl Microbiol Biotechnol, 65*, 520–529.

Neilands, J. B. (1995). Siderophores: Structure and function of microbial iron transport compounds. *The Journal of Biological Chemistry, 270*, 26723-2672.

Nepple, B.B. & Bachofen, R. (1997). Induction of stress proteins in the phototrophic bacterium *Rhodobacter sphaeroides. FEMSMicrobiologyLetters, 153 (1),* 173-180.

Nickel, C.M., Vandekerckhove, J., Beyer, P., Tadros, M. (1997). Molecular analysis of the *Rhodobacter capsulatus* chaperone *dnaKJ* operon: purification and characterization of DnaK. *Gene,192*, 251–259.

Oda, Y., Samanta, S.K., Rey, F.E., Wu, L., Liu, X., Yan., T. et al. (2005).Functional genomic analysis of three nitrogenase isozymes in the photosynthetic bacterium *Rhodopseudomonas palustris. Journal of Bacteriology, 187 (22),* 7784-7794.

Onder, O., Aygun-Sunar, S., Selamoglu, N., Daldal, F. (2010). A glimpse into the proteome of phototrophic bacterium *Rhodobacter capsulatus. Adv. Exp. Med. Biol., 675,* 179-209.

Özgür, E., Mars, A., Peksel, B., Louwerse, A., Yücel, M., Gündüz, U. et al. (2010). Biohydrogen production from beet molasses by sequential dark and photofermentation. *International Journal of Hydrogen Energy, 35 (2*), 511-517.

Özgür, E., Uyar, B., Öztürk, Y., Yücel, M., Gündüz, U., Eroğlu, İ. (2010). Biohydrogen production by *Rhodobacter capsulatus* on acetate at fluctuating temperatures. *Resources, Conservation and Recycling, 54,* 310–314.

Öztürk, Y., Yücel, M., Daldal, F., Mandacı, S., Gündüz, U., Türker, L., Eroğlu, I. (2006). Hydrogen production by using *Rhodobacter capsulatus* mutants with genetically modified electron transfer chains, *International Journal of Hydrogen Energy, 31*, 1545–1552.

Panoff, J.P., Thammavongs, B., Gue´guen, M., Boutibonnes, P. (1998). Cold stress responses in mesophilic bacteria. *Cryobiology, 36*, 75–83.

Pappas, C.T., Sram, J., Moskvin, O.V., Ivanov, P.S., Mackenzie, R.C., Choudhary, M. et al. (2004). Construction and validation of the *Rhodobacter sphaeroides* 2.4.1 DNA microarray: Transcriptome flexibility at diverse growth modes. *Journal of Bacteriology, 186 (14)*, 4748-4758.

Pasternak, C., Chen, W., Heck, C., Klug, G. (1996). Cloning, nucleotide sequence and characterization of the rpoD gene encoding the primary sigma factor of *Rhodobacter capsulatus. Gene, 176 (1-2), 177*-184.

Peng, X., Wood, C.L., Blalock, E.M., Chen, K.C., Landfield, P.W., Stromberg, A.J. (2003). Statistical implications of pooling RNA samples for microarray experiments. *BMC Bioinformatics, 4(26)*, doi:10.1186/1471-2105-4-26.

Phadtare, S. (2004). Recent developments in bacterial cold-shock response. *Curr. Issues Mol. Biol. 6,* 125-136.

Raina, A. & Jänne, J. (1975). Physiology of the natural polyamines putrescine, spermidine and spermine. *Med Biol., 53 (3)*, 121-47.

Reslewic,S., Zhou, S., Place, M., Zhang,Y., Briska, A., Goldstein, S., et al. (2005). Whole-Genome Shotgun Optical Mapping of *Rhodospirillum rubrum*. *Applied and Environmental Microbiology, 71(9)* ,5511-5522.
Ron, E.Z. (2006). Bacterial Stress Response. *Prokaryotes, 2,* 1012-1027.

Sasikala, K., Ramana, C.H., Rao, P.R., (1991). Environmental regulation for optimal biomass yield and photoproduction of hydrogen by *Rhodobacter sphaeroides* O.U.001, *International Journal of Hydrogen Energy, 16*, 597-601.

Schumann, W. (2001). CIRCE, HAIR and ROSE: Regulation of the bacterial heat shock response stress responses. *Biology International, 40*, 3-5.

Sevinç, P. (2010). Kinetic Analyses of the Effects of Temperature and Light Intensity on Growth, Hydrogen Production, and Organic Acid Utilization by *Rhodobacter capsulatus.* M.Sc. Thesis in Biotechnology Engineering Department, Middle East Technical University, Ankara, Turkey.

Shen, G. & Golbeck, J.H. (2006). Assembly of the bound iron–sulfur clusters in Photosystem I. *Advances in Photosynthesis and Respiration, 24*, 529–548.

Shi, L., Jones, W.D., Jensen, V.R., Harris, S.C., Perkins, R.G., Goodsaid, F.M. et al. (2008). The balance of reproducibility, sensitivity, and specificity of lists of differentially expressed genes in microarray studies. *BMC Bioinformatics, 9(10),* doi:10.1186/1471-2105-9-S9-S10.

Shih, J.H., Michalowska, A.M., Dobbin, K., Ye, Y., Qiu, T.H., Green, J.E. (2004). Effects of pooling mRNA in microarray class comparisons. Bioinformatics, 20(18), 3318-3325.

Silva RM, Duarte ICN, Paredes JA, Lima-Costa T, Perrot M, et al. (2009). The yeast *PNC1* longevity gene is up-regulated by mRNA mistranslation. *PLoS ONE, 4(4)*: e5212. doi:10.1371/journal.pone.0005212.

Slonczewski, J.L., Foster, J.W. (2009). *Microbiology an evolving science*. New York: W.W. Norton & Company, Inc.

Soballe, B. & Poole, R.K. (1999). Microbial ubiquinones: multiple roles in respiration, gene regulation and oxidative stress management. *Microbiology, 145*, 1817-1830.

Stokes, J.L. (1944). Substitution of thymine for “folic acid” in the nutrition of lactic acid bacteria. *J. Bacteriol, 48(2)*, 201-209.

Straus, D., Walter, W., Gross, C.A. (1987). The heat shock response of *E.coli* is regulated by changes in the concentration of sigma 32. *Nature, 329*, 348-351.

Strnad, H., Lapidus, A., Paces, J., Ulbrich, P., Vlcek, C., Paces, V., Haselkorn.,R. (2010). Complete genome sequence of the photosynthetic purple nonsulfur bacterium *Rhodobacter capsulatus* SB 1003. *J. Bacteriol*. doi:10.1128/JB.00366-10.
Synder, L & Champness, W. (2007). Molecular Genetics of Bacteria, ASM Press, Washingtn DC, USA.

Tabor, C.W. & Tabor, H. (1976). 1,4-diaminobutane (putrescine), spermidine and spermine. *Annu. Rev. Biochem., 45*, 285-306.

Tonnies, G., Das, D.N., Fen, F. (1966). Pantothenate and coenzyme A in bacterial growth. *Journal of Bacteriology, 92 (3)*, 707-713.

Truglio, J.J., Karakas, E., Rhau, B., Wang, H., DellaVecchia, M.J., Van Houten, B., Kisker, C. (2006). Structural basis for DNA recognition and processing by UvrB. *Nature Structural & Molecular Biology, 13,* 360-364.

Tsuihiji, H., Yamazaki, Y., Kamikubo, H., Imamoto, Y., Kataoka, M. (2006). Cloning and characterization of *nif* structural and regulatory genes in the purple sulfur bacterium, *Halorhodospira halophila. Journal of Bioscience and Bioengineering, 101(3),* 263-270.

Uyar, B., Eroğlu, İ., Yücel, M., Gündüz, U., Türker, L. (2007). Effect of light intensity, wavelength and illumination protocol on hydrogen production in photobioreactors. *International Journal of Hydrogen Energy, 32,* 4670–4677.

Uyar, B. (2008) Hydrogen Production by Microorganisms in Solar Bioreactor, Ph.D. Thesis in Biotechnology Engineering Department, Middle East Technical University, Ankara, Turkey.

Uyar, B., Schumacher, M., Gebicki, J., Modigell, M. (2009). Photoproduction of hydrogen by Rhodobacter capsulatus from thermophilic fermentation effluent. *Bioprocess and Biosystem Engineering, 32*, 603–606.

Ünlü, G., Sevinç, P., Gündüz, U. (2009). Expression Analysis of nif A and nif H genes in *Rhodobacter sphaeroides* O.U.001 at different temperatures, presented in International Symposium on Biotechnology: Developments and Trends, BTEC 2009, METU, Ankara, Turkey.

Weaver, P.F., Wall, J.D., Gest, H. (I975). Characterization of *Rhodopseudomonas capsulata. Arch. Microbiol. 105,* 207-216 .

Wiethaus, J., Wirsing, A., Narberhaus, F, Masepohl, B. (2006). Overlapping and specialized functions of the molybdenum-dependent regulators Mop A and Mop B in *Rhodobacter capsulatus. Journal of bacteriology,188(24)*, 8441-8451.

Yilmaz, R., Yücel, M. and Öktem, H.A. (2008).Quality assessment of gene expression data for an affymetrix platform with the two sample t-tests statistical analysis. *International Journal of Biotechnology & Biochemistry, 4*: 101-108.

Yura, T. (2001). Toward Integration of Bacterial Stress Responses. *Biology International, 40*, 6-7.

Zhang, Y. & Shen, J. (2006). Effect of temperature ans iron concentration on the growth and hydrogen production of mixed bacteria. *International Journal of Hydrogen Energy, 31(4),* 441-446.

Zhou, S., Kvikstad, E., Kile, A., Severin, J., Forrest, D., Runnheim, R. et al. (2003). Whole-genome shotgun optical mapping of *Rhodobacter sphaeroides* strain 2.4.1 and its use for whole-genome shotgun sequence assembly. *Genome Res., 13,* 2142-2151.

APPENDIX A

COMPOSITION OF THE GROWTH AND HYDROGEN PRODUCTION MEDIA

Table A. 1 The constituents of 1 L of MPYE medium

Medium composition	Amount
Bactopeptone	3 g
Yeast extract	3 g
$MgCl_2$	1.6 ml from 1M stock
$CaCl_2$	1 ml form 1M stock

The constituents are dissolved in distilled water; pH is adjusted to 7, and sterilized by autoclaving.

Table A. 2 The constituents of the growth and hydrogen production media per liter of solution

Medium Composition	Growth Medium 20/10 A/G	Hydrogen Production Medium 30/2 A/G
KH_2PO_4	3 g	3 g
$MgSO_4.7H_2O$	0.5 g	0.5 g
$CaCl_2.2H_2O$	0.05 g	0.05 g
Acetic acid	1.15 ml	2.29 ml
Na-Glutamate	1.85 g	0.36 g
Vitamin Solution (10x)	0.1 ml	0.1 ml
Trace Element Solution (10x)	0.1 ml	0.1 ml
Fe-citrate (50x)	0.5 ml	0.5ml

Vitamin solution, trace element solution and Fe-citrate solution are added after the other constituents are dissolved in distilled water and sterilized by autoclaving.

Table A. 3 The composition of 100 ml of 10x vitamin solution

Composition	Amount
Thiamin Chloride Hydrochloride (B1)	500 mg
Niacin (Nicotinic acid)	500 mg
D + Biotin	15 mg

The constituents are dissolved in distilled water and sterilized by filtering

Table A. 4 The composition of 100 ml of 10x trace element solution

Composition	**Amount**
$ZnCl_2$	70 mg
$MnCl_2.4H_2O$	100 mg
H_3BO_3	60 mg
$CoCl_2.6H_2O$	200 mg
$CuCl_2.2H_2O$	20 mg
$NiCl_2.6H_2O$	20 mg
$NaMoO_4.2H_2O$	40 mg
HCl (25% v/v)	1 ml

The constituents are dissolved in distilled water and sterilized by autoclaving.

Ferric citrate solution: 5 g ferric citrate is dissolved in 100 ml distilled water and sterilized by autoclaving

APPENDIX B

OPTICAL DENSITY-DRY CELL WEIGHT CALIBRATION CURVE

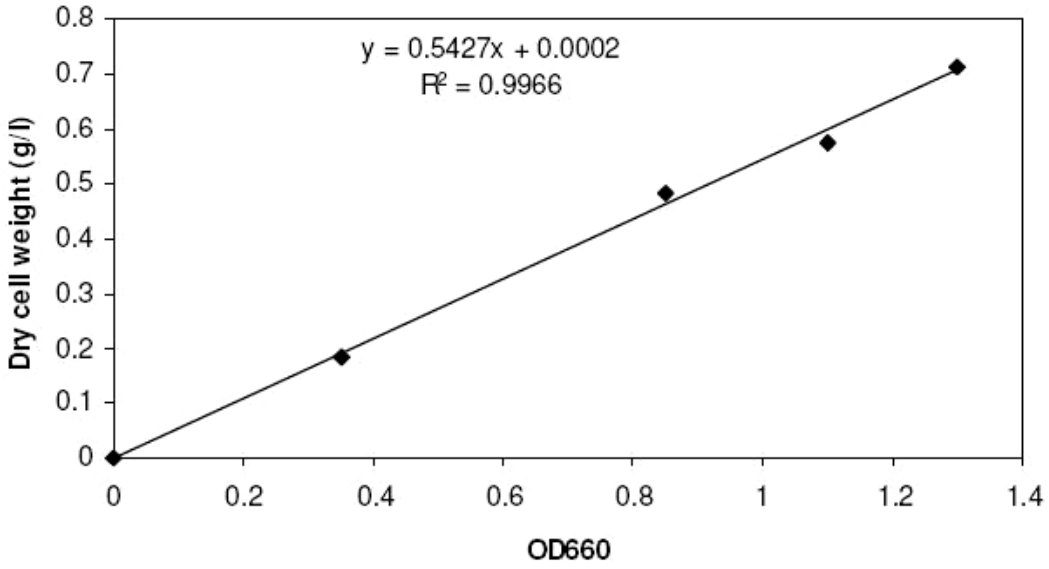
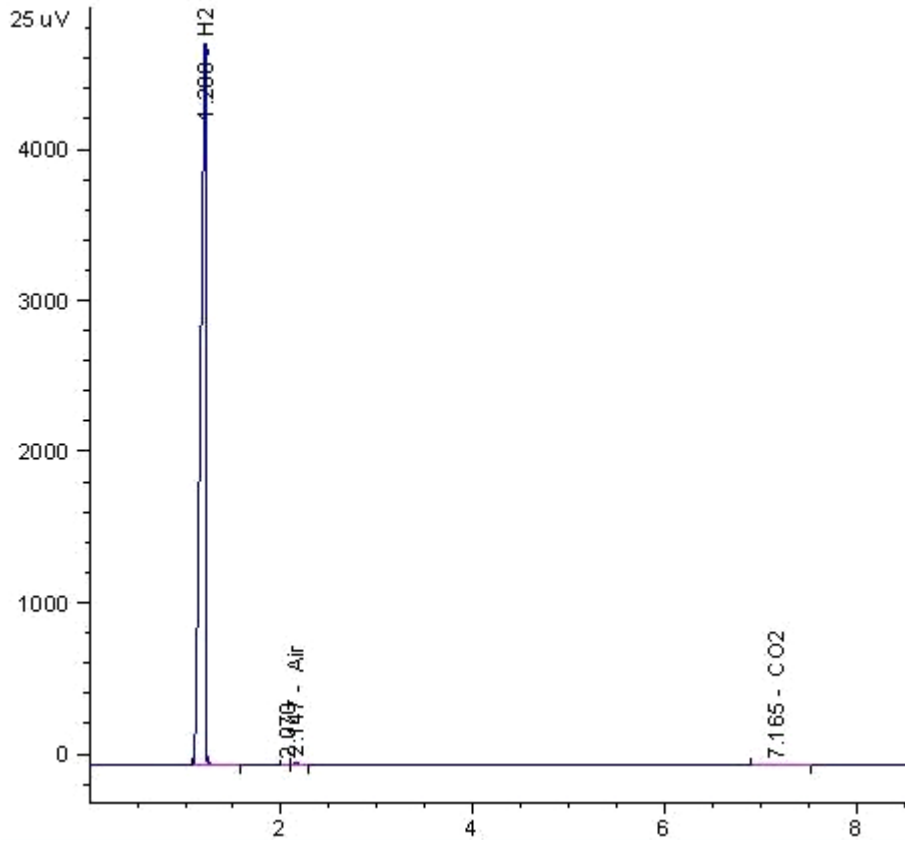


Figure B.1 Calibration curve and the regression trend line for *Rhodobacter capsulatus* (DSM1710) dry cell weight vs OD660nm (Uyar, 2008)

APPENDIX C

SAMPLE GAS CHROMATOGRAM

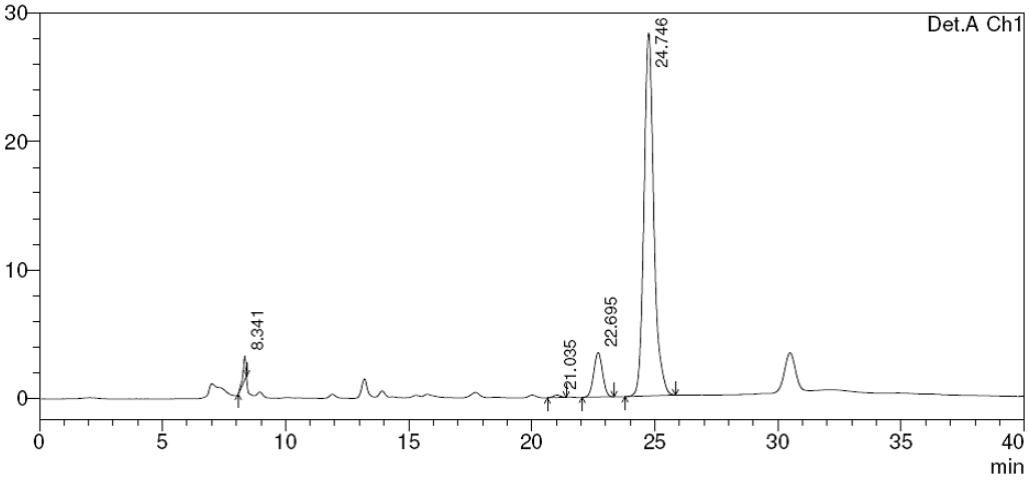


RetTime [min]	Type	Area [25 uV*s]	Amt/Area	Norm %	Grp	Name
1.208	BB	1.78157e4	5.36760e-3	92.790983		H2
2.147	VB	104.16668	4.30476e-2	4.351104		Air
7.165	BB	52.88443	5.56929e-2	2.857913		CO2
Totals :				100.000000		

Figure C. 1 A sample chromatogram for GC analysis of the produced gas

APPENDIX D

SAMPLE HPLC CHROMATOGRAM AND CALIBRATION CURVES



PeakTable

Detector A Ch1 210nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	8.341	14689	1859	1.654	5.516
2	21.035	3735	182	0.420	0.542
3	22.695	86422	3453	9.730	10.248
4	24.746	783359	28200	88.196	83.694
Total		888205	33695	100.000	100.000

Figure D. 1 A sample HPLC chromatogram for organic acids analysis. Peak 1 (mobile phase- H_2SO_4), Peak 2 (lactic acid), Peak 3 (formic acid) and Peak 4 (acetic acid)

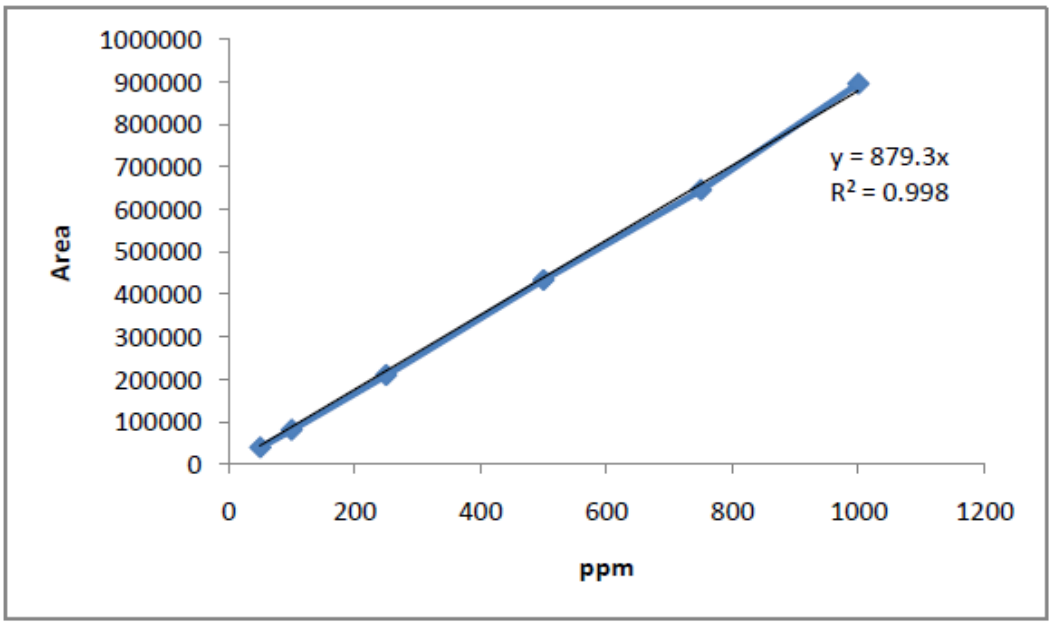


Figure D. 2 The acetic acid calibration curve

APPENDIX E

COMPOSITION OF TRIS-EDTA BUFFER

1 M Tris-HCl (pH 7.5) stock solution: Dissolve 157.6 g Tris-HCl in 1 L of distilled water

1 mM EDTA (pH 8) stock solution: Dissolve 186.1 g EDTA dissodium salt in 700 ml distilled water, adjust pH to 8 by 5N NaOH, bring volume to 1 L.

Table E. 1 The constituents of TE buffer (10mM Tris, 1mM EDTA)

Composition	Amount
1 M Tris-HCl stock solution	1 ml
0.5 M EDTA stock solution	0.2 ml

The constituents are dissolved in 100 mL of distilled water and autoclaved.

APPENDIX F

A SAMPLE NANODROP PROFILE FOR RNA

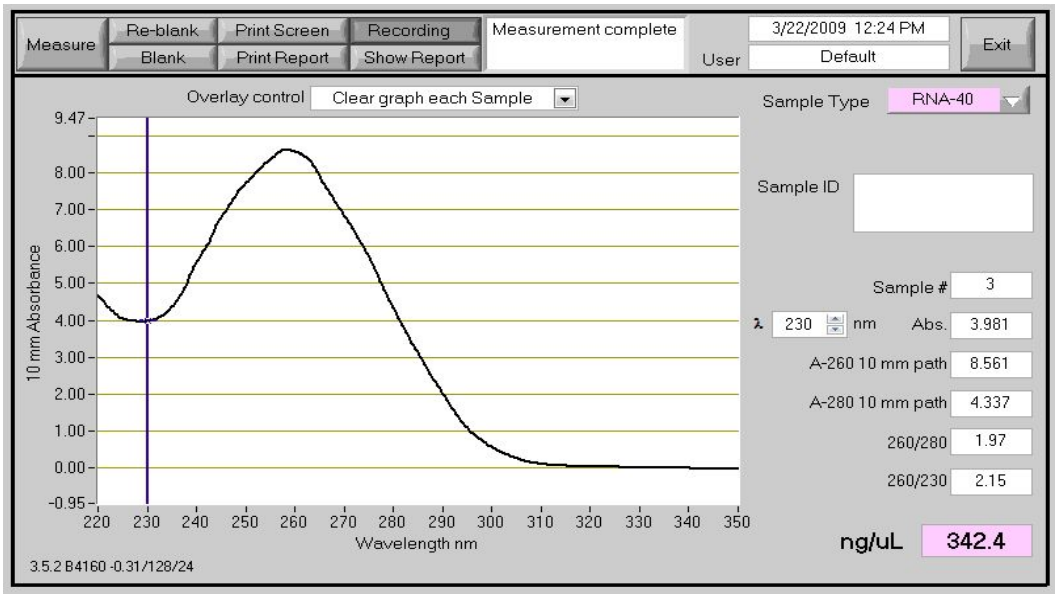


Figure F. 1 A sample NanoDrop profile for a high quality RNA (Cofactor Genomics http://www.cofactorgenomics.com/sample/ Last access: July, 06 2011)

APPENDIX G

A SAMPLE GENECHIP® SCAN IMAGE

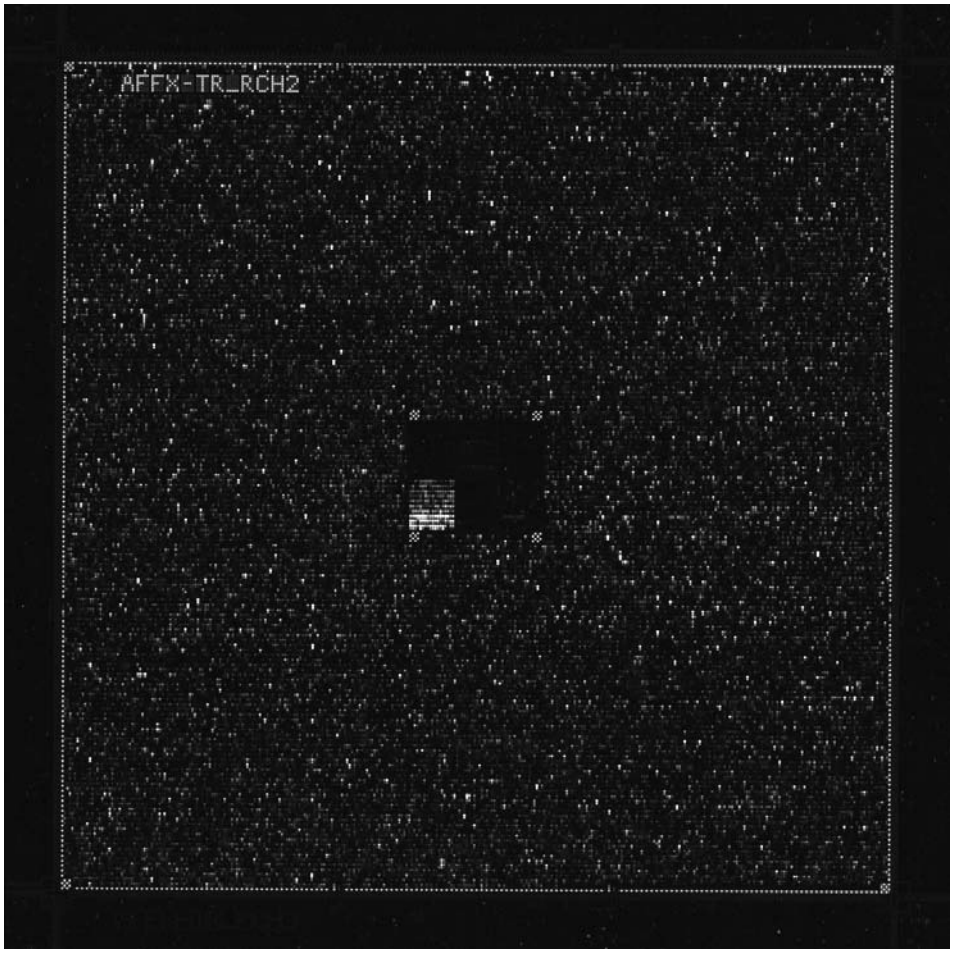


Figure G. 1 The Affymetrix GeneChip® scan image of *R.capsulatus*.

Figure G. 2 The chip name, corner and edges of GeneChip® scan image of *R.capsulatus*

APPENDIX H

THE LISTS OF SIGNIFICANTLY CHANGED GENES OF R. capsulatus UNDER COLD STRESS

Table H. 1 The list of genes of nitrogen metabolism that change significantly under cold stress

Probe Set ID	Gene	Product	Role	2h vs control	Reg.	6h vs control	Reg.	p-value
RCAP_rcc00163_at	gltD	glutamate synthase (NADPH), beta subunit	Glutamate family [Amino acid biosynthesis]	2.290	up	2.213	up	0.0052
RCAP_rcc00566_s_at	nifB	nitrogenase cofactor biosynthesis protein NifB	Nitrogen fixation	6.317	up	2.487	up	0.0204
RCAP_rcc00569_at	nifU	nitrogen fixation protein NifU	Nitrogen fixation	2.663	up	1.232	up	0.0209
RCAP_rcc00570_at	nifK	nitrogenase molybdenum-iron protein beta chain	Nitrogen fixation	29.376	up	16.530	up	0.003
RCAP_rcc00571_at	nifD	nitrogenase molybdenum-iron protein alpha chain	Nitrogen fixation	59.112	up	38.480	up	0.0176
RCAP_rcc00572_at	nifH	nitrogenase iron protein	Nitrogen fixation	99.953	up	44.111	up	0.006
RCAP_rcc00585_at	anfH	nitrogenase iron protein	Nitrogen fixation	33.189	up	20.210	up	0.0873

Table H.1 (continued)

RCAP_rcc00586_at	anfD	nitrogenase iron-iron protein, alpha subunit	Nitrogen fixation	23.486	up	13.126	up	0.0301
RCAP_rcc00587_at	anfG	nitrogenase iron-iron protein, delta subunit	Nitrogen fixation	37.180	up	22.194	up	0.0379
RCAP_rcc01673_at	glnB	nitrogen regulatory protein P-II	Protein interactions	2.756	up	2.093	up	0.0355
RCAP_rcc01674_at	glnA	glutamine synthetase	Glutamate family[Amino acid biosynthesis	2.249	up	2.212	up	0.0027
RCAP_rcc02707_at	cynT	carbonate dehydratase	Inorganic ion transport and metabolism	4.113	down	1.619	down	0.0268
RCAP_rcc03263_at	nifT	NifT/FixU family protein	Nitrogen fixation	11.893	up	4.579	up	0.0028
RCAP_rcc03268_at	nifW	nitrogen fixation protein NifW	Nitrogen fixation	2.624	up	1.387	up	0.0159
RCAP_rcc03269_at	nifV	homocitrate synthase NİFv	Nitrogen fixation	5.267	up	1.789	up	0.0033
RCAP_rcc03270_at	nifS	cysteine desulfurase NifS	Biosynthesis of cofactors, prosthetic groups, and carriers	3.228	up	1.121	up	0.0132
RCAP_rcc03275_at	fdxB	ferredoxin III	Nitrogen fixation	7.582	up	2.965	up	0.0086
RCAP_rcc03277_at		protein of unknown function DUF269	Nitrogen fixation	13.946	up	7.135	up	0.01
RCAP_rcc03278_at	nifX	nitrogen fixation protein NifX	Nitrogen fixation	18.337	up	4.358	up	0.0002

Table H.1 (continued)

RCAP_rcc03279_at	nifN	nitrogenase molybdenum-iron cofactor biosynthesis protein NifN	Nitrogen fixation	6.598	up	1.957	up	0.0038
RCAP_rcc03280_at	nifE	nitrogenase molybdenum-iron cofactor biosynts protein NifE	Nitrogen fixation	3.069	up	1.223	up	0.0089
RCAP_rcc03282_at	apbE	thiamin biosynthesis lipoprotein ApbE	Nitrogen fixation	2.126	up	1.245	up	0.038
RCAP_rcc03287_at	rnfA	electron transport complex protein RnfA	Nitrogen fixation	20.417	up	5.874	up	0.0033
RCAP_rcc03288_at		electron transport complex protein RnfB	Nitrogen fixation	9.373	up	1.972	up	0.0018
RCAP_rcc03289_at		electron transport complex protein RnfC	Nitrogen fixation	17.068	up	7.526	up	0.0036
RCAP_rcc03290_at		electron transport complex protein RnfD	Nitrogen fixation	7.219	up	4.282	up	0.0042
RCAP_rcc03291_at		electron transport complex protein RnfG	Nitrogen fixation	8.091	up	3.526	up	0.0028
RCAP_rcc03292_at		electron transport complex protein RnfE	Nitrogen fixation	7.124	up	5.601	up	0.0039

Table H. 2 The list of genes of photosynthesis that change significantly under cold stress

Probe Set ID	Gene	Product	Role	2h vs control	Reg	6h vs control	Reg	p-value
RCAP_rcc00744_at	atpF	ATP synthase F0, B subunit	ATP-proton motive force interconversion	3.575	up	1.640	up	0.0535
RCAP_rcc01172_at	hemE	uroporphyrinogen decarboxylase	Heme, porphyrin, and cobalamin	2.038	up	1.125	up	0.0030
RCAP_rcc01830_at	fba	fructose-bisphosphate aldolase	Glycolysis/ gluconeogenesis	2.056	up	3.441	up	0.0137
RCAP_rcc01834_at	fbp	fructose-bisphosphatase	Glycolysis/ gluconeogenesis	2.748	up	2.564	up	0.0988
RCAP_rcc03496_at	maeB	malate dehydrogenase (oxaloacetate-decarboxylating) (NADP(+))	Energy metabolism	2.157	up	1.115	up	0.0582

Table H. 3 The list of genes of electron transport that change significantly under cold stress

Probe Set ID	Gene	Product	Role	2h vs control	Reg	6h vs control	Reg	p-value
RCAP_rcc00165_at		NAD-dependent epimerase/dehydratase family protein	Electron transport	2.577	down	1.049	up	0.0189
RCAP_rcc00679_at	crtI	phytoene dehydrogenase		4.495	down	1.585	down	0.0130

Table H.3 (continued)

RCAP_rcc00744_at	atpF	ATP synthase F0, B subunit	ATP-proton motive force interconversion	3.575	up	1.640	up	0.0535
RCAP_rcc00768_at	hupB	hydrogenase, large subunit	Electron transport	1.442	up	3.407	up	0.0749
RCAP_rcc01421_at	fldA	flavodoxin	Electron transport	2.081	up	1.409	up	0.0934
,RCAP_rcc02031_at		protein of unknown function DUF989	Electron transport	2.107	up	1.355	up	0.0581
RCAP_rcc02398_at	gor	glutathione-disulfide reductase	Electron transport	2.365	down	1.517	down	0.0197
RCAP_rcc02517_at	trxC	thioredoxin	Electron transport	2.151	down	2.109	down	0.0476
RCAP_rcc02814_at	trxB	thioredoxin-disulfide reductase	Electron transport	4.734	down	1.791	down	0.0236
RCAP_rcc03085_at	cydA	cytochrome d ubiquinol oxidase, subunit I	Electron transport	2.006	down	1.207	up	0.0714
RCAP_rcc03258_at		cytoChrome c biogenesis protein, transmembrane region	Electron transport	3.240	down	1.124	down	0.0481
RCAP_rcc03265_at		LRV FeS4 cluster domain protein	Electron transport	3.844	up	1.191	up	0.0368

Table H.3 (continued)

RCAP_rcc03284_at	fdxN	ferredoxin I	Domain [Hypothetical proteins]	23.668	up	10.214	up	0.0093
RCAP_rcc03285_at	fdxC	ferredoxin IV	Electron transport	13.582	up	2.455	up	0.0000
RCAP_rcc03286_at	norV	anaerobic nitric oxide reductase flavorubredoxin	Electron transport	3.623	up	1.182	up	0.0156
RCAP_rcc03287_at	rnfA	electron transport complex protein RnfA	Electron transport	20.417	up	5.874	up	0.0033
RCAP_rcc03288_at	rnfB	electron transport complex protein RnfB	Electron transport	9.373	up	1.972	up	0.0018
RCAP_rcc03289_at	rnfC	electron transport complex protein RnfC	Electron transport	17.068	up	7.526	up	0.0036
RCAP_rcc03290_at	rnfD	electron transport complex protein RnfD	Electron transport	7.218	up	4.282	up	0.0042
RCAP_rcc03291_at	rnfG	electron transport complex protein RnfG	Electron transport	8.091	up	3.526	up	0.0028
RCAP_rcc03292_at	rnfE	electron transport complex protein RnfE	Electron transport	7.124	up	5.601	up	0.0039
RCAP_rcc03334_at	trxA	thioredoxin	Electron transport	5.027	down	1.705	down	0.0217

Table H. 4 The list of genes of protein metabolism that change significantly under cold stress

Probe Set ID	Gene	Product	Role	2h vs control	reg	6h vs control	reg	p-value
RCAP_rcc00034_s_at	hslU	ATP-dependent hsl protease ATP-binding subunit hslU	Protein folding and stabilization	5.356	down	3.532	down	0.0979
RCAP_rcc00035_s_at	hslV	ATP-dependent protease HslV	Degradation of proteins, peptides, and glycopeptides	5.836	down	2.666	down	0.0585
RCAP_rcc00207_at	lys1	saccharopine dehydrogenase (NAD+, L-lysine-forming)	Aspartate family [Amino acid biosynthesis]	1.472	up	3.051	up	0.0239
RCAP_rcc00223_at	dnaJ	chaperone DnaJ	Protein folding and stabilization	4.573	down	2.022	down	0.0422
RCAP_rcc00224_at	dnaK	chaperone DnaK	Protein folding and stabilization	5.482	down	1.307	down	0.0031
RCAP_rcc00268_at		peptidase, M16 family	Degradation of proteins, peptides, and glycopeptides	2.960	down	1.105	down	0.0383

Table H.4 (continued)

RCAP_rcc00327_at	rplQ	50S ribosomal protein L17	Ribosomal proteins: synthesis and modification	4.036	up	2.120	up	0.0982
RCAP_rcc00390_at		peptidase, S49 family	Degradation of proteins, peptides, and glycopeptides	2.126	down	1.597	down	0.0349
RCAP_rcc00480_at	rpsU	30S ribosomal protein S21	Ribosomal proteins: synthesis and modification	5.586	up	2.147	up	0.0835
RCAP_rcc00505_at		type II secretion system protein E	Protein and peptide secretion and trafficking	2.315	up	1.552	up	0.0998
RCAP_rcc00507_at		type II secretion system protein	Protein and peptide secretion and trafficking	2.536	up	1.405	up	0.0374
RCAP_rcc00547_at		glutaredoxin family protein	Posttranslational modification, protein turnover, chaperones	4.392	down	1.341	down	0.0307

Table H.4 (continued)

RCAP_rcc00629_at		acetyltransferase, GNAT family	Aromatic amino acid family [Amino acid biosynthesis]	2.001	up	1.006	down	0.0018
RCAP_rcc00762_at	hoxW	hydrogenase maturation factor HowW	Protein modification and repair	2.570	up	1.333	down	0.0078
RCAP_rcc01167_at	clpS	ATP-dependent Clp protease adaptor protein ClpS	Degradation of proteins, peptides, and glycopeptides	3.602	down	1.333	down	0.0108
RCAP_rcc01709_at	ppiA	peptidyl-prolyl cis-trans isomerase A	Protein folding and stabilization	2.466	down	1.257	down	0.0479
RCAP_rcc01731_at		peptidase, U32 family	Degradation of proteins, peptides, and glycopeptides	2.430	down	1.728	down	0.0198
RCAP_rcc01922_at	metA	homoserine O-succinyltransferase	Aspartate family [Amino acid biosynthesis]	2.703	down	1.412	down	0.0542
RCAP_rcc02399_at	hflK	HflK protein	Degradation of proteins, peptides, and glycopeptides	8.354	down	2.421	down	0.0070

Table H.4 (continued)

RCAP_rcc02400_at	hflC	HflC protein	Degradation of proteins, peptides, and glycopeptides	6.670	down	2.875	down	0.0245
RCAP_rcc02477_at	groS	chaperonin GroS	Protein folding and stabilization	6.357	down	1.041	down	0.0021
RCAP_rcc02478_at	groL	chaperonin GroL	Protein folding and stabilization	8.799	down	1.019	down	0.0037
RCAP_rcc02583_at	lon	ATP-dependent protease La	Degradation of proteins, peptides, and glycopeptides	5.656	down	1.884	down	0.0193
RCAP_rcc02609_at	clpP	ATP-dependent Clp protease, ATP-binding subunit ClpX	Degradation of proteins, peptides, and glycopeptides	2.995	down	1.054	down	0.0292
RCAP_rcc02818_at	ibpA	small heat shock protein IbpA	Protein folding and stabilization	13.262	down	1.762	down	0.0023
RCAP_rcc02977_at	clpA	ATP-dependent Clp protease, ATP-binding subunit ClpA	Degradation of proteins, peptides, and glycopeptides	3.889	down	1.271	down	0.0850

Table H.4 (continued)

RCAP_rcc03192_at	ftsH	cell division protease FtsH	Degradation of proteins, peptides, and glycopeptides Posttranslational modification, protein turnover, chaperones	3.232	down	1.401	down	0.0038
RCAP_rcc03259_at	msrB	peptide-methionine (R)-S-oxide reductase	Protein modification and repair	2.083	down	1.012	down	0.0622
RCAP_rcc03406_at	clpB	chaperone ClpB	Protein folding and stabilization	12.622	down	1.735	down	0.0035
RCAP_rcc03446_at	serC	phosphoserine aminotransferase	Serine family Amino acid biosynthesi	3.416	up	2.419	up	0.0029
RCAP_rcc03477_at	msrB	methionine S-oxide reductase (R-form oxidizing)	Posttranslational modification, protein turnover, chaperones	3.166	down	1.221	up	0.0095
RCAP_rcc03478_at	msrA	methionine S-oxide reductase (S-form oxidizing)	Posttranslational modification, protein turnover, chaperones	2.327	down	1.193	up	0.0349

Table H. 5 The list of genes of cell enveleope metabolism that change significantly under cold stress

Probe Set ID	Gene	Product	Role	2h vs control	reg	6h vs control	reg	p-value
RCAP_rcc00083_at		membrane protein, putative	Other [Cell envelope]	2.033	up	1.370	up	0.0378
RCAP_rcc00184_at		outer membrane efflux protein	Cell wall/membrane/envelope biogenesis	2.030	up	1.245	up	0.0035
RCAP_rcc00373_at	mdoH	glucans biosynthesis glucosyltransferase H	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	4.527	down	1.843	down	0.0506
RCAP_rcc00433_at		efflux transporter, RND family, MFP subunit	Cell envelope	2.322	down	1.721	down	0.0543
RCAP_rcc00604_at		integral membrane protein, TerC family	Cell envelope	4.349	down	1.537	down	0.0644
RCAP_rcc00802_at	mreC	rod shape-determining protein MreC	Biosynthesis and degradation of murein sacculus and peptidoglycan	2.772	down	1.515	down	0.0967
RCAP_rcc00812_at		protein of unknown function UPF0118, transmembrane	Cell envelope	2.747	down	1.533	down	0.0278

Table H.5 (continued)

RCAP_rcc00871_at		protein of unknown function UPF0005, transmembrane	Cell envelope	10.034	down	1.740	down	0.0013
RCAP_rcc01181_at		membrane protein, putative	Cell envelope	5.376	down	1.619	down	0.0204
RCAP_rcc01281_at		membrane protein, putative	Cell envelope	3.024	down	1.005	down	0.0153
RCAP_rcc01551_at	bcp	peroxiredoxin	Cell envelope	2.996	down	1.029	up	0.0326
RCAP_rcc01622_at		UPF0114, transmembrane	Cell envelope	6.665	down	2.136	down	0.0107
RCAP_rcc01713_at		lipoprotein, putative	Cell envelope	2.194	down	1.045	down	0.0099
RCAP_rcc01725_at		protein of unknown function DUF475, transmembrane	Cell envelope	4.229	down	1.577	down	0.0257
RCAP_rcc01739_at		membrane protein, putative	Cell envelope	2.375	down	1.052	down	0.0164
RCAP_rcc01746_at		protein of unknown function DUF485, transmembrane	Cell envelope	2.185	up	1.387	up	0.0925
RCAP_rcc01818_at		protein of unknown function DUF485, transmembrane	Cell envelope	2.786	down	1.935	down	0.0789

Table H.5 (continued)

RCAP_rcc01872_at	lpxD	UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	2.652	down	1.499	down	0.0668
RCAP_rcc02137_at		membrane protein, putative	Cell envelope	2.626	down	1.611	down	0.0463
RCAP_rcc02138_at		protein of unknown function DUF1212, transmembrane	Cell envelope	3.126	down	1.615	down	0.0617
RCAP_rcc02148_at		lipid A biosynthesis acyltransferase	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	2.096	down	1.753	down	0.0559
RCAP_rcc02176_at		membrane protein, putative	Cell envelope	3.719	down	1.611	down	0.0107
RCAP_rcc02251_at		membrane protein, putative	Cell envelope	2.011	up	1.039	up	0.0896
RCAP_rcc02401_at		membrane protein, putative	Cell envelope	4.639	down	2.622	down	0.0395
RCAP_rcc02507_at		membrane protein, putative	Cell envelope	4.939	down	2.074	down	0.0198

Table H.5 (continued)

RCAP_rcc02994_at	exoD	exopolysaccharide synthesis, ExoD	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	2.716	down	2.045	down	0.0343
RCAP_rcc03220_at		protein of unknown function DUF697, transmembrane	Cell envelope	2.705	down	1.591	down	0.0759
RCAP_rcc03399_at		membrane protein, putative	Cell envelope	3.342	down	1.927	down	0.0223
RCAP_rcc03402_at		membrane protein, putative	Cell envelope	2.310	down	1.418	down	0.0108
RCAP_rcc03454_at		protein of unknown function YGGT, transmembrane	Cell envelope	3.917	down	1.572	down	0.0242
RCAP_rcc03468_at	pncA	pyrazinamidase/nicotinamidase	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	3.031	down	1.073	down	0.0608
RCAP_rcp00059_at		methyltransferase, type 12 family	Cell wall/membrane/envelope biogenesis	2.267	down	1.035	up	0.0193

Table H. 6 The list of genes of lipid metabolism that change significantly under cold stress

Probe Set ID	Gene	Product	Role	2h vs control	Reg	6h vs control	Reg	p-value
RCAP_rcc00236_at	accD	acetyl-CoA carboxylas carboxyl transferase beta subunit	Biosynthesis [Fatty acid and phospholipid metabolism	3.307	down	1.121	down	0.0165
RCAP_rcc00342_at		oxidoreductase, short-chain dehydrogenase/reductase family	Lipid transport and metabolism	2.618	down	1.491	down	0.0991
RCAP_rcc00442_at	prpE	propionate--CoA ligase	Fatty acid and phospholipid metabolism	2.250	up	1.040	down	0.0023
RCAP_rcc01678_at	acpP	acyl carrier protein	Biosynthesis [Fatty acid and phospholipid metabolism	3.545	up	1.294	up	0.0998
RCAP_rcc02795_at		sterol-binding domain protein	Lipid transport and metabolism	2.113	up	1.011	up	0.0477
RCAP_rcc03165_at	accA	acetyl-CoA carboxylase, carboxyl transferase alpha subunit	Biosynthesis [Fatty acid and phospholipid metabolism	3.303	down	2.033	down	0.0636

Table H.6 (continued)

RCAP_rcc03179_at	phbB	acetoacetyl-CoA reductase	Biosynthesis Fatty acid and phospholipid metabolism	2.664	up	1.002	down	0.0081
RCAP_rcc03423_at	ispH	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	Lipid transport and metabolism, Cell wall/membrane/envelope biogenesis	2.256	down	1.425	down	0.0207

Table H. 7 The list of genes of nucleic acid metabolism that change significantly under cold stress

Probe Set ID	Gene	Product	Role	2h vs control	reg	6h vs control	Reg	p-value
RCAP_rcc00201_at		site-specific DNA-methyltransferase (adenine-specific)	Replication, recombination and repair	2.578	up	1.070	up	0.0041
RCAP_rcc00263_at		two component transcriptional regulator, winged helix family	DNA interactions	5.642	down	1.774	down	0.0693
RCAP_rcc00284_at	pnp	polyribonucleotide nucleotidyltransferase	Degradation of RNA	6.457	up	2.964	up	0.0211

Table H.7 (continued)

RCAP_rcc00328_at		autoinducer-binding transcriptional regulator, LuxR family	DNA interactions	2.294	up	1.039	down	3.8631E-4
RCAP_rcc00561_at	mopA	molybdenum transport operon repressor MopA	DNA interactions	2.673	up	1.150	up	0.0027
RCAP_rcc00568_at	rpoN	RNA polymerase sigma-54 factor	Transcription factors	6.031	up	2.602	up	0.0269
RCAP_rcc00651_at		two component transcriptional regulator, winged helix family	Transcription	2.166	up	1.168	down	0.0330
RCAP_rcc00811_at	hda	DnaA-homolog protein Hda	DNA replication, recombination, and repair	1.099	down	2.365	up	0.0829
RCAP_rcc01384_at	uvrB	UvrABC system protein B	DNA replication, recombination, and repair	4.826	down	1.988	down	0.0136
RCAP_rcc01561_at		transcriptional regulator, Crp/Fnr family	DNA interactions	3.988	down	2.155	down	0.0211
RCAP_rcc01618_at	rrmJ	ribosomal RNA large subunit methyltransferase J	tRNA and rRNA base modification	2.459	down	1.713	down	0.0213

Table H.7 (continued)

RCAP_rcc01661_at	trmU	tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase	tRNA and rRNA base modification	2.711	down	1.572	down	0.0443
RCAP_rcc01801_at	hfq	RNA chaperone Hfq	Other [Regulatory functions]	2.452	up	1.020	down	0.0017
CAP_rcc02001_at		peptidoglycan-binding domain 1 protein	Prophage functions [Mobile and extrachromosomal element functions]	2.03769	up	1	down	0.0058
RCAP_rcc02165_at	rne	ribonuclease E	RNA processing	3.679	up	1.004	down	0.0014
RCAP_rcc02195_at		HNH endonuclease family protein	Restriction/modification	2.672	down	2.773	down	0.0836
RCAP_rcc02298_at		acetyltransferase, GNAT family	Translation, ribosomal structure and biogenesis	3.125	down	1.430	down	0.0550
RCAP_rcc02515_at	rhlE	ATP-dependent RNA helicase RhlE	Other [Transcription]	1.579	down	2.145	down	0.0855
RCAP_rcc02527_at		transcriptional regulator, XRE family	Other [Regulatory functions]	2.422	up	1.077	up	0.0941
RCAP_rcc02584_at	hup	DNA-binding protein HU	Chromosome-associated proteins	2.249	up	1.092	down	0.0096
RCAP_rcc02590_at	dksA	DnaK suppressor protein	DNA interactions	2.190	up	1.038	down	0.0955
RCAP_rcc02813_at	lrp	leucine-responsive regulatory protein	DNA interactions	2.870	up	1.176	down	0.0068

Table H.7 (continued)

RCAP_rcc02922_at		phage virion morphogenesis protein	Prophage functions [Mobile and extrachromosomal element functions]	3.591	down	2.682	down	0.0607
RCAP_rcc02958_at	phrB	deoxyribodipyrimidine photo-lyase	DNA replication, recombination, and repair	2.417	down	1.972	down	0.0049
RCAP_rcc03055_at	dnaG	DNA primase	DNA replication, recombination, and repair	2.225	down	1.839	down	0.0027
RCAP_rcc03145_at		transcriptional regulator, AsnC/Lrp family	DNA interactions	2.142	up	1.037	up	0.0855
RCAP_rcc03147_at		transcriptional regulator, MerR family	DNA interactions	2.564	up	1.109	down	0.0557
RCAP_rcc03383_at		transcriptional regulator, MarR family	DNA interactions	2.889	down	1.235	up	0.0228
RCAP_rcc03407_at	dinB	DNA polymerase IV	DNA replication, recombination, and repair	2.302	down	2.135	down	0.0728
RCAP_rcp00031_at		phage integrase	Prophage functions [Mobile and extrachromosomal element functions]	2.843	down	1.355	down	0.0980

Table H. 8 The list of genes of transporters and binding proteins that change significantly under cold stress

Probe Set ID	Gene	Product	Role	2h vs control	Reg	6h vs control	Reg	p-value
RCAP_rcc00017_at	xylH	xylose ABC transporter, permease protein XylH	Carbohydrates, organic alcohols, and acids [Transport and binding proteins]	2.074	down	1.011	down	0.0820
RCAP_rcc00563_at	modB	molybdenum ABC transporter, permease protein ModB	Anions [Transport and binding proteins]	3.113	up	1.602	up	0.0033
RCAP_rcc00619_at		ABC transporter, permease protein	Unknown substrate	2.776	down	1.198	down	0.0297
RCAP_rcc00624_at		amino acid ABC transporter, periplasmic amino acid-binding protein	Amino acids, peptides and amines [Transport and binding proteins]	2.489	up	1.835	up	0.0478
RCAP_rcc00627_at		amino acid ABC transporter, ATP-binding protein	Amino acids, peptides and amines [Transport and binding proteins]	2.205	up	1.176	up	0.0024
RCAP_rcc00879_at	phnA	protein PhnA	Inorganic ion transport and metabolism [Metabolism]	2.697	up	1.104	up	0.0123
RCAP_rcc01120_at	metK	methionine adenosyltransferase	Coenzyme transport and metabolism [Metabolism]	2.060	up	2.181	up	0.0082

Table H.8 (continued)

RCAP_rcc01177_at		ABC transporter ATP-binding protein	Unknown substrate [Transport and binding proteins]	2.444	down	1.669	down	0.0449
RCAP_rcc01178_at		protein of unknown function DUF214, permase predicted	Unknown substrate [Transport and binding proteins]	2.447	down	1.313	down	0.0123
RCAP_rcc01451_at		efflux transporter, RND family, MFP subunit	Unknown substrate [Transport and binding proteins]	2.112	down	1.612	down	0.0891
RCAP_rcc01545_at	dctM	TRAP C4-dicarboxylate transport system permease, DctM subunit	Carbohydrates, organic alcohols, and acids [Transport and binding proteins]	1.871	up	2.110	up	0.0503
RCAP_rcc01589_at		ErfK/YbiS/YcfS/YnhG family protein	Unknown substrate [Transport and binding proteins]	3.690	down	1.251	up	0.0842
RCAP_rcc01615_at		methylenetetrahydrofolate reductase family protein Enzymes of unknown specificity	Amino acid transport and metabolism [Metabolism]	4.002	down	2.332	down	0.0549
RCAP_rcc01617_at	gppA	guanosine-5'-triphosphate,3'-diphosphate pyrophosphatase	Nucleotide transport and metabolism [Metabolism],Inorganic ion transport and metabolism	2.521	down	1.622	down	0.0476

Table H.8 (continued)

RCAP_rcc01853_at	livG	branched-chain amino acid ABC transporter, ATP-binding protein LivG	Amino acids, peptides and amines [Transport and binding proteins]	2.098	up	1.331	up	0.0284
RCAP_rcc01857_at	livM	branched-chain amino acid ABC transporter, permease protein	Amino acids, peptides and amines [Transport and binding proteins]	2.067	up	1.091	up	0.0024
RCAP_rcc02120_at		ABC transporter, ATP-binding/periplasmic substrate-binding protein	Unknown substrate [Transport and binding proteins]	2.853	down	2.051	down	6.9365E-4
RCAP_rcc02127_at	phaG	monovalent cation/proton antiporter, G subunit	Cations and iron carrying compounds [Transport and binding proteins]	2.788	down	2.369	down	0.0685
RCAP_rcc02128_at	phaF	monovalent cation/proton antiporter, F subunit	Cations and iron carrying compounds [Transport and binding proteins]	3.175	down	2.366	down	0.0632
RCAP_rcc02129_at	phaE	monovalent cation/proton antiporter, E subunit	Cations and iron carrying compounds [Transport and binding proteins]	3.635	down	1.804	down	0.0258

Table H.8 (continued)

RCAP_rcc02130_at	phaD	monovalent cation/proton antiporter, D subunit	Cations and iron carrying compounds [Transport and binding proteins]	2.663	down	1.786	down	0.0605
RCAP_rcc02131_at	phaC	monovalent cation/proton antiporter, C subunit	Cations and iron carrying compounds [Transport and binding proteins]	2.771	down	1.887	down	0.0722
RCAP_rcc02132_at	phaAB	monovalent cation/proton antiporter, A/B subunit	Cations and iron carrying compounds [Transport and binding proteins]	4.591	down	1.875	down	0.0195
RCAP_rcc02190_at		heavy metal translocating P-type ATPase	Cations and iron carrying compounds [Transport and binding proteins]	3.637	up	1.527	up	0.0306
RCAP_rcc02218_at	modB	molybdate ABC transporter, permease protein ModB	Anions [Transport and binding proteins]	2.114	up	1.749	up	0.0168
RCAP_rcc02223_at		ABC transporter, permease protein	Unknown substrate [Transport and binding proteins]	2.050	down	1.271	down	0.0195
RCAP_rcc02526_at	amaB	N-carbamoyl-L-amino acid amidohydrolase	Amino acid transport and metabolism [Metabolism]	3.007	up	1.341	up	0.0441
RCAP_rcc02578_at		iron(III) ABC transporter periplasmic iron(III)-compound-binding protein	Cations and iron carrying compounds [Transport and binding proteins]	2.333	down	2.125	up	0.0479

Table H.8 (continued)

RCAP_rcc02579_at		Fe(III) ABC transporter, permease protein	Cations and iron carrying compounds [Transport and binding proteins]	2.910	down	1.127	down	0.0021
RCAP_rcc03023_at	dctQ	tripartite ATP-independent periplasmic transporter, DctQ component	Amino acids, peptides and amines [Transport and binding proteins]	3.477	down	1.261	up	0.0358
RCAP_rcc03024_at	dctP	TRAP dicarboxylate transporter, DctP subunit	Carbohydrates, organic alcohols, and acids [Transport and binding proteins]	2.214	down	1.723	up	0.0314
RCAP_rcc03119_at		ion transport 2 family protein	Amino acids, peptides and amines [Transport and binding proteins]	2.059	down	1.185	down	0.0698
RCAP_rcc03120_at		ABC transporter, ATP-binding protein	Amino acids, peptides and amines [Transport and binding proteins],	4.799	down	1.413	down	0.0085
RCAP_rcc03139_at		ABC transporter, permease/ ATP-binding protein	Unknown substrate [Transport and binding proteins]	2.608	down	1.806	down	0.0791

Table H.8 (continued)

RCAP_rcc03146_at	gabT	4-aminobutyrate aminotransferase	Amino acid transport and metabolism [Metabolism]	4.030	up	1.102	up	0.0465
RCAP_rcc03384_at	emrA	multidrug resistance protein A	Unknown substrate [Transport and binding proteins] Defense mechanisms [Cellular processes and signaling]	2.237	down	1.157	up	0.0157
RCAP_rcc03385_at	emrB	multidrug resistance protein B	Unknown substrate [Transport and binding proteins]	2.176	down	1.199	up	0.0674
RCAP_rcp00044_at		iron siderophore/cobalamin ABC transporter, ATP-binding protein	Cations and iron carrying compounds [Transport and binding proteins]	2.174	up	1.383	up	0.0508
RCAP_rcp00050_at		iron siderophore/cobalamin ABC transporter, periplasmic iron siderophore/cobalamin-binding protein	Cations and iron carrying compounds [Transport and binding proteins]	2.591	up	1.291	down	0.0520
RCAP_rcp00056_at		nitrate/sulfonate/bicarbonate ABC transporter, ATP-binding protein	Anions [Transport and binding proteins]	7.154	down	1.994	down	0.0681

Table H. 9 The list of genes of biosynthesis of cofactors, prostethic groups and carriers that change significantly under cold stress

Probe Set ID	Gene	Product	Role	2h vs control	Reg	6h vs control	Reg	p-value
RCAP_rcc00071_at	nadA	quinolinate synthetase A	Pyridine nucleotides [Biosynthesis of cofactors, prosthetic groups, and carriers]	2.158	down	1.079	down	0.0150
RCAP_rcc00210_at	ubiG	3-demethylubiquinone-9 3-O-methyltransferase	Menaquinone and ubiquinone [Biosynthesis of cofactors, prosthetic groups, and carriers]	2.425	down	1.280	down	0.05712
RCAP_rcc00622_at	moeA	molybdopterin biosynthesis protein MoeA	Molybdopterin [Biosynthesis of cofactors, prosthetic groups, and carriers]	2.009	up	1.046	up	0.0085
RCAP_rcc00628_at	menC	O-succinylbenzoic acid synthetase	Menaquinone and ubiquinone [Biosynthesis of cofactors, prosthetic groups, and carriers]	2.258	up	1.118	down	0.0209
RCAP_rcc01210_at	folB	dihydroneopterin aldolase	Folic acid [Biosynthesis of cofactors, prosthetic groups, and carriers]	2.341	down	1.773	down	0.0217

Table H.9 (continued)

RCAP_rcc01231_at		ubiquinone biosynthesis hydroxylase, UbiH/UbiF/VisC/COQ6 family	Menaquinone and ubiquinone [Biosynthesis of cofactors, prosthetic groups, and carriers]	5.003	down	1.768	down	0.0434
RCAP_rcc01449_at	ispG	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	Other [Biosynthesis of cofactors, prosthetic groups, and carriers]	2.666	down	1.540	down	0.0677
RCAP_rcc01599_at	iscA	iron-sulfur cluster assembly accessory protein	Other [Biosynthesis of cofactors, prosthetic groups, and carriers]	2.550	down	1.725	down	0.0293
RCAP_rcc01614_at	metH	methionine synthase, B subunit	Folic acid [Biosynthesis of cofactors, prosthetic groups, and carriers]	2.836	down	1.700	down	0.0189
RCAP_rcc02325_at		HesB/YadR/YfhF family protein	Other [Biosynthesis of cofactors, prosthetic groups, and carriers]	4.670	down	1.776	down	0.0445
RCAP_rcc02419_at		dihydroneopterin aldolase	Folic acid [Biosynthesis of cofactors, prosthetic groups, and carriers]	2.624	down	1.336	down	0.0279

Table H.9 (continued)

RCAP_rcc03274_at	nifQ	NifQ family protein	Other [Biosynthesis of cofactors, prosthetic groups, and carriers]	5.229	up	1.500	up	0.0494
RCAP_rcp00134_at	citG	triphospho ribosyl-dephospho-CoA synthase	Pantothenate and coenzyme A [Biosynthesis of cofactors, prosthetic groups, and carriers]	2.427	up	1.025	up	0.0476

APPENDIX I

THE LISTS OF SIGNIFICANTLY CHANGED GENES OF *R. capsulatus* UNDER HEAT STRESS

Table I. 1 The list of genes of nitrogen metabolism that change significantly under heat stress

Probe Set ID	Gene	Product	Role	2h vs control	Reg	6h vs control	Reg	p-value
RCAP_rcc00163_at	gltD	glutamate synthase (NADPH), beta subunit	Glutamate family [Amino acid biosynthesis]	5.815	up	3.265	up	0.0492
RCAP_rcc01674_at	glnA	glutamine synthetase	Glutamate family [Amino acid biosynthesis]	5.476	down	9.266	down	0.0013
RCAP_rcc02529_at		pyridine nucleotide disulphide oxidoreductase family protein	Enzymes of unknown specificity	2.511	down	2.022	down	0.0336
RCAP_rcc03387_at	glnB	nitrogen regulatory protein P-II	Protein interactions	2.532	down	7.445	down	0.0912

Table I. 2 The list of genes of photosynthesis that change significantly under heat stress

Probe Set ID	Gene	Product	Role	2h vs control	Reg	6h vs control	Reg	p-value
RCAP_rcc00484_at	ald	alanine dehydrogenase	Amino acids and amines [Energy metabolism]	2.293	down	1.424	down	0.0886
RCAP_rcc00660_at	pucC	PucC protein	Photosynthesis	2.221	down	1.728	down	0.0855
RCAP_rcc00690_at	pufQ	cytochrome, subunit PufQ	Photosynthesis]	3.008	up	1.584	up	0.0744
RCAP_rcc00693_at	pufL	photosynthetic reaction center, L subunit	Photosynthesis	1.894	down	3.864	down	0.0825
RCAP_rcc00694_at	puf M	photosynthetic reaction center, M subunit	Photosynthesis	2.156	down	3.102	down	0.0691
RCAP_rcc00721_at	sucD	succinyl-CoA synthetase (ADP-forming), alpha subunit	TCA cycle	2.870	down	3.659	down	0.0822
RCAP_rcc00733_at	sdhA	succinate dehydrogenase, flavoprotein subunit	TCA cycle	1.982	down	2.147	down	0.0089
RCAP_rcc00736_at	sdhB	succinate dehydrogenase, iron-sulfur subunit	TCA cycle	2.558	down	3.508	down	0.0324

Table I.2 (continued)

RCAP_rcc01835_at	cbbR	RuBisCO operon transcriptional regulator CbbR	DNA interactions [Regulatory functions]	2.006	up	1.775	up	0.0642
RCAP_rcc01887_at	icd	Isocitrate dehydrogenase (NADP(+))	TCA cycle	2.934	down	3.828	down	0.0088
RCAP_rcc02150_at	acnA	aconitate hydratase	TCA cycle	2.482	down	2.801	down	0.0930
RCAP_rcc02531_at	pucA	light-harvesting protein B-800/850, alpha chain	Photosynthesis	1.070	up	2.053	down	0.0405
RCAP_rcc02970_at	atpC	ATP synthase F1, epsilon subunit	ATP-proton motive force interconversion	1.981	down	2.559	down	0.0380
RCAP_rcc02971_at	atpD	ATP synthase F1, beta subunit	ATP-proton motive force interconversion	6.198	down	9.121	down	0.0211
RCAP_rcc03062_at	glpX	fructose-1,6-bisphosphatase	Pentose phosphate pathway	2.256	up	1.098	up	0.0631

Table I. 3 The list of genes of electron transport that change significantly under heat stress

Probe Set ID	Gene	Product	Role	2h vs control	Reg	6h vs control	Reg	p-value
RCAP_rcc00036_s_at	trxA	thioredoxin	Electron transport]	2.198	up	1.741	up	0.0895
RCAP_rcc00143_at	etfA	electron transfer flavoprotein, alpha subunit	Central intermediary metabolism	2.093	down	2.427	down	0.0196
RCAP_rcc00144_at	etfB	electron transfer flavoprotein, beta subunit	Central intermediary metabolism	2.732	down	4.266	down	0.0355
RCAP_rcc00573_at	fdxD	ferredoxin V	Electron transport	3.345	down	2.949	down	0.0075
RCAP_rcc00690_at	pufQ	cytochrome, subunit PufQ	Electron transport	3.008	up	1.584	up	0.0744
RCAP_rcc00693_at	pufL	photosynthetic reaction center, L subunit	Electron transport	1.894	down	3.864	down	0.0825
RCAP_rcc00694_at	pufM	photosynthetic reaction center, M subunit	Electron transport	2.156	down	3.102	down	0.0691
RCAP_rcc00715_at	pntA	pyridine nucleotide transhydrogenase, alpha subunit	Electron transport	1.943	down	3.761	down	0.0216

Table I.3 (continued)

RCAP_rcc00733_at	sdhA	succinate dehydrogenase, flavoprotein subunit	TCA cycle	1.982	down	2.147	down	0.0089
RCAP_rcc00736_at	sdhB	succinate dehydrogenase, iron-sulfur subunit	TCA cycle	2.558	down	3.508	down	0.0324
RCAP_rcc00740_at	atpI	ATP synthase F0, I subunit	ATP-proton motive force interconversion	1.858	down	2.430	down	0.0279
RCAP_rcc00767_at	hupA	hydrogenase, small subunit	Electron transport	2.436	down	3.333	down	0.0778
RCAP_rcc00768_at	hupB	hydrogenase, large subunit	Electron transport	3.744	down	5.254	down	0.0249
RCAP_rcc00769_at	hupC	hydrogenase, cytochrome b subunit	Electron transport	2.401	down	2.354	down	0.0387
RCAP_rcc00772_at	hupG	hydrogenase expression/formation protein HupG	Electron transport	1.756	up	2.226	up	0.0856
RCAP_rcc00785_at	sqr	sulfide:quinone oxidoreductase	Electron transport	2.345	down	3.155	down	0.0097
RCAP_rcc01159_at	ccoQ	cytochrome c oxidase, Cbb3-type, subunit IV	Electron transport	2.372	down	2.563	down	0.0408
RCAP_rcc01160_at	ccoP	cytochrome c oxidase, Cbb3-type, subunit III	Electron transport	2.806	down	2.771	down	0.0611

Table I.3 (continued)

RCAP_rcc01240_at	cycA	cytochrome c2	Electron transport	2.530	down	3.014	down	0.0335
RCAP_rcc01517_at	nuoA	NADH-quinone oxidoreductase, A subunit	Electron transport	2.363	down	2.214	down	0.0795
RCAP_rcc01518_at	nuoB	NADH-quinone oxidoreductase, B subunit	Electron transport	2.401	down	2.791	down	0.0719
RCAP_rcc01519_at	nuoC	NADH-quinone oxidoreductase, C subunit	Electron transport	2.884	down	2.758	down	0.0153
RCAP_rcc01520_at	nuoD	NADH-quinone oxidoreductase, D subunit	Electron transport	3.101	down	4.132	down	0.0043
RCAP_rcc01521_at	nuoE	NADH-quinone oxidoreductase, E subunit	Electron transport	2.700	down	2.869	down	0.0289
RCAP_rcc01527_at	nuoG	NADH-quinone oxidoreductase, G subunit	Electron transport	1.720	down	2.262	down	0.0553
RCAP_rcc01529_at	nuoH	NADH-quinone oxidoreductase, H subunit	Electron transport	2.118	down	1.663	down	0.0749
RCAP_rcc02607_at		NADH ubiquinone oxidoreductase subunit, NDUFA12 family	Electron transport	2.086	down	2.055	down	0.0722
RCAP_rcc02679_at		pyridine nucleotide disulphide oxidoreductase family protein	Electron transport	3.398	down	2.822	down	0.0088

Table I.3 (continued)

RCAP_rcc02769_at	petB	ubiquinol--cytochrome-c reductase, cytochrome b subunit	Electron transport	2.555	down	3.819	down	0.0377
RCAP_rcc02970_at	petC	ATP synthase F1, epsilon subunit	ATP-proton motive force interconversion	1.981	down	2.559	down	0.0380
RCAP_rcc02971_at	atpD	ATP synthase F1, beta subunit	ATP-proton motive force interconversion	6.198	down	9.121	down	0.0211
RCAP_rcc02972_at	atpG	ATP synthase F1, gamma subunit	ATP-proton motive force interconversion]	5.359	down	8.073	down	0.0531
RCAP_rcc03258_at		cytoChrome c biogenesis protein, transmembrane region	Electron transport	2.297	down	1.989	down	0.0981

Table I. 4 The list of genes of protein metabolism that change significantly under heat stress

Probe Set ID	Gene	Product	Role	2h vs control	Reg	6h vs control	Reg	p-value
RCAP_rcc00187_at	hslO	chaperonin HslO	Protein folding and stabilization	3.809	up	3.549	up	0.0371

Table I.4 (continued)

RCAP_rcc00218_at	argJ	arginine biosynthesis bifunctional protein ArgJ	Glutamate family [Amino acid biosynthesis]	1.618	down	2.266	down	0.0918
RCAP_rcc00228_at		oxidoreductase, DSBA family	Posttranslational modification, protein turnover, chaperones	2.530	down	2.471	down	0.0986
RCAP_rcc00267_at		peptidase, M16 family	Degradation of proteins, peptides, and glycopeptides	3.310	down	3.382	down	0.0947
RCAP_rcc00268_at		peptidase, M16 family	Degradation of proteins, peptides, and glycopeptides	3.963	down	2.210	down	0.0271
RCAP_rcc00321_at	rplO	50S ribosomal protein L15	Ribosomal proteins: synthesis and modification	8.431	up	8.222	up	5.4674 E-4
RCAP_rcc00350_at	rpmH	50S ribosomal protein L34	Ribosomal proteins: synthesis and modification	4.232	up	3.551	up	0.0134
RCAP_rcc00361_at	rpmE	50S ribosomal protein L31	Ribosomal proteins: synthesis and modification	3.899	up	2.197	up	0.0983
RCAP_rcc00480_at	rpsU	30S ribosomal protein S21	Ribosomal proteins: synthesis and modification	12.697	up	4.962	up	0.0026

Table I.4 (continued)

RCAP_rcc00617_at		secretion protein, HlyD family	Protein and peptide secretion and trafficking	2.617	down	1.666	down	0.0931
RCAP_rcc00770_at	hupD	hydrogenase maturation protease HupD	Protein modification and repair	4.131	up	5.417	up	0.0152
RCAP_rcc01167_at	clpS	ATP-dependent Clp protease adaptor protein ClpS	Degradation of proteins, peptides, and glycopeptides	1.291	up	2.704	up	0.0839
RCAP_rcc01183_at	hisB	imidazoleglycerol-phosphate dehydratase	Histidine family Amino acid biosynthesis	2.704	up	2.422	up	0.0751
RCAP_rcc01632_at	yaeT	outer membrane protein assembly factor YaeT	Protein and peptide secretion and trafficking	1.702	down	2.028	down	0.0442
RCAP_rcc01909_at	rpmF	50S ribosomal protein L32	Ribosomal proteins: synthesis and modification	6.984	up	5.084	up	0.0092
RCAP_rcc02299_at	rpmA	50S ribosomal protein L27	Ribosomal proteins: synthesis and modification	2.563	up	2.672	up	0.0891
RCAP_rcc02400_at	hflC	HflC protein	Degradation of proteins, peptides, and glycopeptides	3.551	down	3.161	down	0.0504

Table I.4 (continued)

RCAP_rcc02506_at	rluB	ribosomal large subunit pseudouridine synthase B	tRNA and rRNA base modification	5.304	up	3.032	up	0.0661
RCAP_rcc02583_at	lon	ATP-dependent protease La	Degradation of proteins, peptides, and glycopeptides Posttranslational modification, protein turnover, chaperones	4.331	down	2.544	down	0.0235
RCAP_rcc02608_at	clpX	ATP-dependent Clp protease, ATP-binding subunit ClpX	Protein folding and stabilization Degradation of proteins, peptides, and glycopeptides, Posttranslational modification, protein turnover, chaperones	3.159	down	3.731	down	0.0376
RCAP_rcc02609_at	clpP	ATP-dependent Clp protease, ATP-binding subunit ClpX	Degradation of proteins, peptides, and glycopeptides Posttranslational modification, protein turnover, chaperones, Intracellular trafficking, secretion, vesicular transport	3.859	down	2.358	down	0.0676

Table I.4 (continued)

RCAP_rcc02979_at	rpsD	30S ribosomal protein S4	Ribosomal proteins: synthesis and modification	4.736	up	4.148	up	0.0250
RCAP_rcc03230_at	gcp	peptidase, M22 family, glycoprotease	Degradation of proteins, peptides, and glycopeptides Posttranslational modification, protein turnover, chaperones	1.150	up	2.238	up	0.0772
RCAP_rcc03257_at	msrA	peptide-methionine-(S)-S-oxide reductase	Posttranslational modification, protein turnover, chaperones	2.180	down	2.467	down	0.0683
RCAP_rcc03259_at	msrB	peptide-methionine (R)-S-oxide reductase	Protein modification and repair, Adaptations to atypical conditions Posttranslational modification, protein turnover, chaperones	2.491	down	1.920	down	0.0054
RCAP_rcc03406_at	clpB	chaperone ClpB	Protein folding and stabilization Posttranslational modification, protein turnover, chaperones	2.502	down	2.580	down	0.0120
RCAP_rcc03409_at	rpmJ	50S ribosomal protein L36	Ribosomal proteins: synthesis and modification	4.007	up	2.947	up	0.0275

Table I.4 (continued)

RCAP_rcc03494_at	grpE	GrpE protein	Protein folding and stabilization Posttranslational modification, protein turnover, chaperones	2.716	up	2.585	up	0.0689

Table I. 5 The list of genes of cell envelope metabolism that change significantly under heat stress

Probe Set ID	Gene	Product	Role	2h vs control	Reg	6h vs control	Reg	p-value
RCAP_rcc00353_at		membrane protein, putative	Cell envelope	2.913	up	3.264	up	0.0113
RCAP_rcc00372_at		membrane protein, putative	Cell envelope	2.198	down	1.928	down	0.0529
RCAP_rcc00417_at		protein of unknown function DUF6, transmembrane	Cell envelope	2.246	down	1.217	up	0.0502
RCAP_rcc00535_at		membrane protein, putative	Cell envelope	2.050	up	1.269	up	0.0525
RCAP_rcc00786_at		basic membrane lipoprotein family	Cell envelope	2.383	down	2.215	down	0.0664
RCAP_rcc01179_at		transglycosylase, Slt family	Biosynthesis and degradation of murein sacculus and peptidoglycan	1.923	down	2.222	down	0.0580

Table I.5 (continued)

RCAP_rcc01181_at		membrane protein, putative	Cell envelope	2.186	down	1.025	up	0.0520
RCAP_rcc01232_at		membrane protein, putative	Cell envelope]	2.080	down	1.244	down	0.0252
RCAP_rcc01233_at		membrane protein, putative	Cell envelope	5.015	up	4.106	up	0.0033
RCAP_rcc01281_at		membrane protein, putative	Cell envelope	4.250	down	1.165	up	0.0030
RCAP_rcc01560_at		membrane protein, putative	Cell envelope	2.686	down	3.740	down	0.0037
RCAP_rcc01713_at		lipoprotein, putative	Cell envelope	2.719	up	4.679	Up	0.0420
RCAP_rcc01877_at		membrane protein, putative	Cell envelope	3.546	down	2.771	down	0.0195
RCAP_rcc02108_at		membrane protein, putative	Cell envelope	2.320	down	1.108	down	0.0570
RCAP_rcc02172_at		protein of unknown function DUF471, transmembrane	Cell envelope	1.804	up	3.053	up	0.0817
RCAP_rcc02251_at		membrane protein, putative	Cell envelope	2.330	up	2.045	up	0.0645
RCAP_rcc02380_at	ftsI	peptidoglycan synthetase FtsI	Biosynthesis and degradation of murein sacculus and peptidoglycan	2.279	down	2.546	down	0.0851

Table I.5 (continued)

RCAP_rcc02401_at		membrane protein, putative	Cell envelope	2.852	down	2.174	down	0.0360
RCAP_rcc02507_at		membrane protein, putative	Cell envelope	2.378	down	1.305	down	0.0885
RCAP_rcc02962_at		rhamnan synthesis protein F family	Surface structures, wall/membrane/envelope biogenesis	2.093	up	1.241	up	0.0495
RCAP_rcc03030_at		YiaA/B two helix domain family	Cell envelope	2.417	down	1.196	up	0.0054
RCAP_rcc03103_at		protein of unknown function UPF0104, transmembrane	Cell envelope	2.079	down	1.031	down	0.0181
RCAP_rcc03402_at		membrane protein, putative	Cell envelope	2.453	down	1.237	down	0.0397
RCAP_rcc03468_at	pncA	pyrazinamidase/nicotinamidase	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	1.883	up	3.543	up	0.0243

Table I. 6 The list of genes of lipid metabolism that change significantly under heat stress

Probe Set ID	Gene	Product	Role	2h vs control	Reg	6h vs control	Reg	p-value
RCAP_rcc00028_at	idi	isopentenyl-diphosphate delta-isomerase	Lipid transport and metabolism	4.085	up	2.891	up	0.0973
RCAP_rcc00511_at	ispE	4-diphosphocytidyl-2C-methyl-D-erythritol kinase	Lipid transport and metabolism	2.146	down	2.118	down	0.0529
RCAP_rcc00559_at	pmtA	phosphatidylethanolamine N-methyltransferase	Biosynthesis [Fatty acid and phospholipid metabolism]	3.117	up	2.723	up	0.0809
RCAP_rcc01676_at	fabG	3-oxoacyl-[acyl-carrier-protein] reductase	Biosynthesis [Fatty acid and phospholipid metabolism]	2.370	up	1.264	up	0.0165
RCAP_rcc01678_at	acpP	acyl carrier protein	Biosynthesis [Fatty acid and phospholipid metabolism]	4.132	up	1.487	up	0.0863
RCAP_rcc01702_at	cdsA	phosphatidate cytidylyltransferase	Biosynthesis [Fatty acid and phospholipid metabolism]	2.641	down	1.116	down	0.0067

Table I. 7 The list of genes of nucleic acid metabolism that change significantly under heat stress

Probe Set ID	Gene	Product	Role	2h vs control	Reg	6h vs control	Reg	p-value
RCAP_rcc00201_at		site-specific DNA-methyltransferase (adenine-specific)	Replication, recombination and repair	2.231	up	1.070	down	0.0013
RCAP_rcc00249_at	hup	DNA-binding protein HU	Chromosome-associated proteins	4.421	up	1.955	up	0.0227
RCAP_rcc00286_at	nusG	transcription antitermination protein NusG	Transcription factors	3.113	up	2.461	up	0.0678
RCAP_rcc00328_at		autoinducer-binding transcriptional regulator, LuxR family	DNA interactions	2.311	down	3.174	down	0.0519
RCAP_rcc00349_at	rnpA	ribonuclease P	RNA processing	2.065	up	1.919	up	0.0393
RCAP_rcc00458_at	rpoH	RNA polymerase sigma-32 factor	Transcription factors	3.361	down	1.667	down	0.0032
RCAP_rcc00494_at		transcriptional regulator, AsnC/Lrp family	DNA interactions	2.959	up	7.802	up	0.0515

Table I.7 (continued)

RCAP_rcc00602_at	mntR	transcriptional regulator MntR	Regulatory functions	2.371	up	1.856	up	0.0582
RCAP_rcc00611_at	nikR	nickel-responsive regulator NikR	DNA interactions	2.111	up	1.993	up	0.0383
RCAP_rcc01308_at		transposase, IS66 family	NULL	2.119	up	1.199	up	0.0334
RCAP_rcc01309_at		transposase, IS66 family	NULL	2.703	up	1.263	up	0.0341
RCAP_rcc01384_at	uvrB	UvrABC system protein B	DNA replication, recombination, and repair	3.635	down	3.011	down	0.0008
RCAP_rcc01751_at	recA	RecA protein	DNA replication, recombination, and repair	1.895	up	2.357	up	0.0845
RCAP_rcc01904_at		transcriptional regulator, AsnC/Lrp family	DNA interactions	2.226	up	1.706	up	0.0467
RCAP_rcc01982_at		HNH endonuclease	Prophage functions DNA ,replication, recombination, and repair	2.028	up	1.361	up	0.0384

Table I.7 (continued)

RCAP_rcc02007_at		phage integrase	Prophage functions	2.101	up	1.069	up	0.0607
RCAP_rcc02101_at		transposase, IS66 family	Transposon functions	3.266	up	1.073	up	0.0929
RCAP_rcc02115_at		transcriptional regulator, AsnC/Lrp family	DNA interactions	2.405	up	2.441	up	0.0671
RCAP_rcc02191_at		transcriptional regulator, MerR family	DNA interactions	1.991	up	2.381	up	0.0358
RCAP_rcc02195_at		HNH endonuclease family protein	Restriction/modification	2.748	down	3.628	down	0.0792
RCAP_rcc02272_at		transcriptional regulator, LysR family	DNA interactions	2.215	up	2.143	up	0.0316
RCAP_rcc02324_at	dgt	deoxyguanosinetriphosphate triphosphohydrolase	Nucleotide and nucleoside interconversions	1.839	up	3.070	up	0.0061
RCAP_rcc02902_s_at		integrase, catalytic region	NULL	2.030	up	1.004	down	0.0191
RCAP_rcc03054_at	rpoD	RNA polymerase sigma factor RpoD	DNA replication, recombination, and repair;DNA-dependent RNA polymerase	2.919	down	2.406	down	0.0329

Table I.7 (continued)

RCAP_rcc03147_at		transcriptional regulator, MerR family	DNA interactions	2.191	up	1.878	up	0.0089
RCAP_rcc03409_at	rpmJ	50S ribosomal protein L36	Ribosomal proteins: synthesis and modification	4.007	up	2.947	up	0.0275
RCAP_rcc03469_at	pncB	nicotinate phosphoribosyltransferase	Salvage of nucleosides and nucleotides	1.176	down	2.930	up	0.0111
RCAP_rcp00031_at		phage integrase	Prophage functions	7.831	down	5.668	down	0.0114

Table I. 8 The list of genes of trasnporters and binding proteins that change significantly under heat stress

Probe Set ID	Gene	Product	Role	2h vs control	Reg	6h vs control	Reg	p-value
RCAP_rcc00017_at	xylF	xylose ABC transporter, permease protein XylH	Carbohydrates, organic alcohols, and acids [Transport and binding proteins]	2.813	down	2.221	down	0.0373

Table I.8 (continued)

RCAP_rcc00018_at	xylR	xylose ABC transporter, xylose-binding protein XylF	Carbohydrates, organic alcohols, and acids [Transport and binding proteins]	5.273	down	2.717	down	0.0413
RCAP_rcc00089_at		cation diffusion facilitator family transporter	Cations and iron carrying compounds [Transport and binding proteins]	2.299	down	1.335	down	0.0214
RCAP_rcc00092_at	feoB	ferrous iron transport protein B	Cations and iron carrying compounds [Transport and binding proteins]	2.362	down	1.524	down	0.0338
CAP_rcc00111_at	fhuE	outer membrane ferric siderophore receptor	Cations and iron carrying compounds [Transport and binding proteins]	2.007	up	1.321	up	0.0185
RCAP_rcc00335_at	bztA	glutamate/ aspartate ABC transporter, periplasmic glutamate/ aspartate-binding protein BztA	Amino acids, peptides and amines [Transport and binding proteins]	6.171	down	5.969	down	0.0026

Table I.8 (continued)

RCAP_rcc00618_at		ABC transporter, ATP-binding/permease protein	Amino acids, peptides and amines [Transport and binding proteins]	2.944	down	2.984	down	0.0372
RCAP_rcc00619_at		ABC transporter, permease protein	Amino acids, peptides and amines [Transport and binding proteins]	2.645	down	2.599	down	0.0737
RCAP_rcc00706_at	oppA	oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA	Amino acids, peptides and amines [Transport and binding proteins]	3.203	down	3.085	down	0.0069
RCAP_rcc01178_at		protein of unknown function DUF214, permase predicted	Unknown substrate [Transport and binding proteins]	2.196	down	1.654	down	0.0569
RCAP_rcc01199_at	phnL	phosphonates transport ATP-binding protein PhnL	Amino acids, peptides and amines [Transport and binding proteins]	2.482	up	1.245	up	0.0452
RCAP_rcc01243_at	potA	polyamine ABC transporter, ATP binding protein PotA	Amino acids, peptides and amines [Transport and binding proteins]	8.289	down	10.273	down	0.0073

Table I.8 (continued)

RCAP_rcc01244_at	potD	polyamine ABC transporter, periplasmic polyamine-binding protein PotD	Amino acids, peptides and amines [Transport and binding proteins]	14.108	down	15.842	down	0.0014
RCAP_rcc01245_at	potB	polyamine ABC transporter, permease protein PotB	Amino acids, peptides and amines [Transport and binding proteins]	4.566	down	7.072	down	0.0040
RCAP_rcc01246_at	potI	polyamine ABC transporter, permease protein PotI	Amino acids, peptides and amines [Transport and binding proteins]	3.940	down	4.112	down	0.0304
RCAP_rcc01376_at	dctP	TRAP dicarboxylate transporter, DctP subunit	Amino acids, peptides and amines [Transport and binding proteins]	1.538	down	2.436	down	0.0186
RCAP_rcc01647_at		ABC transporter, periplasmic substrate-binding protein	Unknown substrate [Transport and binding proteins]	2.037	up	1.160	up	0.0472

Table I.8 (continued)

RCAP_rcc01852_at	livK	branched-chain amino acid ABC transporter, periplasmic branched-chain amino acid-binding protein LivK	Amino acids, peptides and amines [Transport and binding proteins]	5.693	down	6.933	down	0.0179
RCAP_rcc01895_at	potH	spermidine/putrescine ABC transporter, permease protein PotH	Amino acids, peptides and amines [Transport and binding proteins]	2.010	down	1.706	down	0.0134
RCAP_rcc02036_at	cbiN	cobalt transport protein CbiN	Cations and iron carrying compounds [Transport and binding proteins]	2.419	up	1.484	up	0.0209
RCAP_rcc02127_at	phaG	monovalent cation/proton antiporter, G subunit	Cations and iron carrying compounds [Transport and binding proteins]	2.509	down	2.786	down	0.0675
RCAP_rcc02128_at	phaF	monovalent cation/proton antiporter, F subunit	Cations and iron carrying compounds [Transport and binding proteins]	2.562	down	1.933	down	0.0846
RCAP_rcc02129_at	phaE	monovalent cation/proton antiporter, E subunit	Cations and iron carrying compounds [Transport and binding proteins]	2.732	down	2.191	down	0.0220

Table I.8 (continued)

RCAP_rcc02183_at	potG	spermidine/putrescine ABC transporter, ATP-binding protein PotG	Amino acids, peptides and amines [Transport and binding proteins]	3.135	down	2.833	down	0.0203
RCAP_rcc02184_at	potH	spermidine/putrescine ABC transporter, permease protein PotH	Amino acids, peptides and amines [Transport and binding proteins]	2.976	down	2.301	down	0.0588
RCAP_rcc02186_at	potF	spermidine/putrescine ABC transporter, periplasmic spermidine/putrescine-binding protein PotF	Amino acids, peptides and amines [Transport and binding proteins]	4.010	down	4.508	down	0.0193
RCAP_rcc02223_at		ABC transporter, permease protein	Unknown substrate [Transport and binding proteins]	2.432	down	1.282	down	0.0558
RCAP_rcc02417_at	dctP	TRAP dicarboxylate transporter, DctP subunit	Amino acids, peptides and amines [Transport and binding proteins]	2.687	down	2.326	down	0.0171

Table I.8 (continued)

RCAP_rcc02521_at		pyrimidine ABC transporter, periplasmic pyrimidine-binding protein	Nucleosides, purines and pyrimidines [Transport and binding proteins]	2.719	down	2.890	down	0.0466
RCAP_rcc02522_at		pyrimidine ABC transporter, permease protein	Nucleosides, purines and pyrimidines [Transport and binding proteins]	2.277	down	2.307	down	0.0707
RCAP_rcc02578_at		iron(III) ABC transporter, periplasmic iron(III)-compound-binding protein	Cations and iron carrying compounds [Transport and binding proteins]	3.595	down	1.890	down	0.0735
RCAP_rcc02773_at	hisP	polar amino acid ABC transporter, ATP-binding protein HisP	Amino acids, peptides and amines [Transport and binding proteins]	1.771	down	2.135	down	0.0641
RCAP_rcc02774_at	hisJ	polar amino acid ABC transporter, periplasmic polar amino acid-binding protein HisJ	Amino acids, peptides and amines [Transport and binding proteins]	2.848	down	4.302	down	0.0228

Table I.8 (continued)

RCAP_rcc02959_at	potD	spermidine/putrescine ABC transporter, periplasmic spermidine/putrescine-binding protein	Amino acids, peptides and amines [Transport and binding proteins]	5.240	down	5.187	down	0.0021
RCAP_rcc03023_at	dctQ	tripartite ATP-independent periplasmic transporter, DctQ component	Amino acids, peptides and amines [Transport and binding proteins]	3.824	down	3.008	down	0.0039
RCAP_rcc03024_at	dctP	TRAP dicarboxylate transporter, DctP subunit	Carbohydrates, organic alcohols, and acids [Transport and binding proteins]	10.165	down	9.539	down	0.0015
RCAP_rcc03139_at		ABC transporter, permease/ATP-binding protein	Unknown substrate [Transport and binding proteins]	2.095	down	2.549	down	0.0821
RCAP_rcc03252_at	cydD	cysteine ABC transporter, permease/ATP-binding protein CydD	Amino acids, peptides and amines [Transport and binding proteins]	2.108	down	1.514	down	0.0232

Table I.8 (continued)

RCAP_rcc03297_at		glycine betaine/L-proline ABC transporter, periplasmic glycine betaine/L-proline-binding protein	Amino acids, peptides and amines [Transport and binding proteins]	2.212	up	1.223	up	0.0335
RCAP_rcc03386_at	amtB	ammonium transporter	Cations and iron carrying compounds [Transport and binding proteins]	5.084	down	6.079	down	0.0420
RCAP_rcp00050_at		iron siderophore/cobalamin ABC transporter, periplasmic iron siderophore/cobalamin-binding protein	Cations and iron carrying compounds [Transport and binding proteins]	7.681	up	3.978	up	0.0239

Table I. 9 The list of genes of biosynthesis of cofactors, prosthetic groups and carriers that change significantly under heat stress

Probe Set ID	Gene	Product	Role	2h vs control	Reg	6h vs control	Reg	p-value
RCAP_rcc00456_at	coaBC	bifunctional phosphopantothenoylcysteine decarboxylase/phosphopantothenate--cysteine ligase	Pantothenate and coenzyme A [Biosynthesis of cofactors, prosthetic groups, and carriers]	2.14494	down	2.00969	down	0.0039
RCAP_rcc01614_at	metH	methionine synthase, B subunit	Folic acid [Biosynthesis of cofactors, prosthetic groups, and carriers]	3.01734	down	3.32226	down	1.0390E-4
RCAP_rcc01878_at	sufD	FeS assembly protein SufD	Other [Biosynthesis of cofactors, prosthetic groups, and carriers]	4.36366	down	4.2245	down	0.0306
RCAP_rcc01879_at	sufC	FeS assembly ATPase SufC	Other [Biosynthesis of cofactors, prosthetic groups, and carriers]	6.59808	down	6.35157	down	2.1582E-4
RCAP_rcc02419_at		dihydropteorin aldolase	Folic acid [Biosynthesis of cofactors, prosthetic groups, and carriers]	2.28174	down	1.49579	down	0.0767
RCAP_rcc03433_at	paaK	phenylacetate--CoA ligase	Other [Biosynthesis of cofactors, prosthetic groups, and carriers]	2.15568	up	1.0292	down	0.0083

Table I.9 (continued)

RCAP_rcp00134_at	citG	triphospho ribosyl-dephospho-CoA synthase	Pantothenate and coenzyme A [Biosynthesis of cofactors, prosthetic groups, and carriers]	2.29512	up	1.35027	up	0.0543