

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE
ENGINEERING AND TECHNOLOGY

**EVOLUTIONARY ENGINEERING OF *Rhodobacter sphaeroides* UNDER
SELECTIVE CoCl₂ STRESS**

M.Sc. THESIS

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Department of Molecular Biology – Genetics & Biotechnology

Molecular Biology - Genetics & Biotechnology Programme

Thesis Advisor: Prof. Dr. Zeynep Petek ÇAKAR

JANUARY 2015

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

**EVİRİMSSEL MÜHENDİSLİK YÖNTEMİ İLE *Rhodobacter sphaeroides*'in CoCl₂
STRESİNE DİRENÇLİ SUŞLARININ ELDESİ**

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To my yellow bike,

FOREWORD

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Hana KAMER

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ABBREVIATIONS

ALA	: 5-aminolevulinic acid
CDW	: Cell Dry Weight
cyt	: Cytochrome
DNA	: Deoxyribo Nucleic Acid
DSMZ	: Deutsche Sammlung von Mikroorganismen und Zellkulturen
EDTA	: Ethylenediaminetetraacetic acid
ETC	: Electron Transport Chain
ICM	: Intracytoplasmic Membrane
LHC	: Light Harvesting Complex
ln	: Natural Logarithm
M27	: Medium 27
nm	: Nanometer
OD	: Optical Density
ORF	: Open Reading Frame
PS	: Photosynthesis
RC	: Reaction Center

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EVOLUTIONARY ENGINEERING OF *Rhodobacter sphaeroides* UNDER SELECTIVE CoCl₂ STRESS

SUMMARY

Rhodobacter sphaeroides is a metabolically diverse organism which is able to fix molecular nitrogen and produce H₂ as side product. It can grow under both oxic and anoxic conditions heterotrophically or photosynthetically. This wide repertoire of metabolism puts this organism in the centre of metabolic regulation and bioremediation studies. *R. sphaeroides* is used in the production of vitamin B₁₂, coenzyme Q10, 5-aminolevulinic acid (ALA), porphyrin, as well as in bioremediation of heavy metals. *R. sphaeroides* follows different metabolic strategies when exposed to different stress factors. It is able to regulate its photosynthetic machinery sensing O₂ tensions. When partial O₂ pressure is reduced, *R. sphaeroides* develops invagination in its cellular membrane. Biosynthesis of chromatophores, which are crucial for photosynthesis, takes place in these invaginations. There are three systems involved in regulation of photosynthetic genes: the PrrBA two subunit activation system, the PpsR repressor and the FnrL, anaerobic regulator. The genes are regulated mainly by O₂ levels. *R. sphaeroides* has two chromosomes and five naturally occurring plasmids. Many of its genes are duplicated between the two chromosomes. These gene duplications generate the genetic diversity which allows the bacteria widen its spectrum of metabolic capabilities. Chromosome I contains 3106 open reading frames whereas chromosome 2 contains 874. It possesses a flagellum similar to that of *E. coli* which is able to rotate only in clockwise direction. When exposed to heavy metal stress, *R. sphaeroides* alters fatty acid composition to increase its membrane integrity. The accumulation of heavy metals in the environments imposes new strategies for restoring the original site conditions. Bioremediation, providing high specificity and versatility has received a great attention in this area. In this study, *R. sphaeroides* was used to obtain a cobalt-resistant strain. Evolutionary engineering approach of metabolic engineering was used to increase the cobalt tolerance of the bacterium, by growing it under gradually increasing stress conditions. This approach being a 'bottom-up' approach does not require extensive information about the metabolism of the organism. The *R. sphaeroides* strain R-26 was used in this study. Initial cobalt concentration to be used for selection was determined after screening analysis of the reference strain at 11 different cobalt concentrations. According to the screening results, the selection of the populations was started with 0.1 mM cobalt concentration. Cobalt concentrations were increased gradually from 0.1 mM to 15 mM through 64 generations. Survival rates were monitored every 24 hours by measuring OD₅₃₅ values of control and stress cultures. At the same time points, cultures were spread on LB plates to confirm that no contamination has occurred. Cultures which were grown on solid medium were examined morphologically. Cultures that produced carotenoids were identified by their characteristic red colour. When the survival rate decreased to 0.2, the selection process was terminated for further analysis. Growth behaviour of the reference strain and the last population obtained by evolutionary engineering were

compared in media with and without cobalt. The cultures showed similar growth characteristics when there was no cobalt in the medium. However, there was a significant difference, when cultures were grown in a medium containing 4 mM cobalt. The 64th population cells were able to maintain their growth rate nearly the same when exposed to 4 mM cobalt, while the reference strain's growth was inhibited. These results indicate the improved resistance of 64th population towards cobalt.

EVİRİSEL MÜHENDİSLİK YÖNTEMİ İLE *Rhodobacter sphaeroides*'in CoCl₂ STRESİNE DİRENÇLİ SUŞLARININ ELDESİ

ÖZET

Rhodobacter sphaeroides metabolik çeşitliliğe sahip bir gram negatif α -proteobakteridir. Bu bakteri değişik şartlar altında kullanıldığında karbon kaynağına bağlı olarak fotoheterotrofik, kemoheterotrofik, fototrofik, kemoototrofik ve fermentatif olarak üreyebilmektedir. Havadaki serbest azotu bağlayarak organik azot ihtiyacını karşılar. Kükürtü elektron alıcı olarak kullanacak metabolik aktiviteye sahip değilken hidrojeni indirgeyici güç olarak kullanabilmektedir. Bu özelliğinden dolayı *R. sphaeroides* organik hidrojen üretiminde kullanılmaktadır. Fotosentetik metabolizması O₂ varlığına bağlı olarak düzenlenir. Kısmi O₂ basıncının düşük olduğu şartlarda *R. sphaeroides* hücre membranında invajinasyon oluşturarak fotosentez için gerekli olan kromatoforların biyosentezi için ortam sağlar.

Fotosentezde rol alan genlerin regülasyonundan üç sistem sorumludur: PrrBA - 2 alt birimli aktivasyon sistemi, PpsR – baskılayıcı sistem ve FnrL anaerobik regülatörü. cbb3 sitokrom c oksidaz ve koenzim Q havuzu, oksijen varlığında oksijen seviyelerini ölçerek oluşturdukları sinyalleri PrrBA sistemine ve PpSR baskılayıcısına yönlendirir. FnrL anaerobik enerji üretiminden sorumlu olan genleri aktive ederek aerobik üremeden anaerobik üremeye geçişi sağlar. Prp (fotosentetik cevap düzenleyici) sistemi iki bileşenden oluşur: membranda yer alan kinaz yapısındaki PrrB sensörü ve onun tamamlayıcısı olan cevap düzenleyici PrrA. Bu sistem anaerobik şartlar altında fotosentetik genleri aktifleştirir. PrrBA sistemi CO₂, N₂ ve H₂ kullanımında görev alan genlerin regülasyonundan sorumludur. Aerobik şartlarda, şiddetli ışık altında fotosentetik gen grubunda yer alan PsSR redoks proteini baskılayıcı olarak rol alır. *R. sphaeroides* 2 dairesel kromozom ve 5 endojen plazmid içermektedir. Kromozomlar sırası ile 3.188.631 ve 943.022 baz çifti büyüklüğündedir. Plazmidlerin 4 tanesi yaklaşık 4 kb iken, en küçük olan 42 kb dir. Kromozom 1; 3106, kromozom 2; 874 açık okuma çerçevesi içerir. Bu organizmanın genomu çok sayıda DNA dizi kopyaları içermektedir. Bu dizi kopyalarının *R. sphaeroides*'in geniş spektrumlu metabolik aktivitesinden büyük ölçüde sorumlu olduğu düşünülmektedir.

R. sphaeroides lateral uzantılı bir kamçıya sahiptir. Bu kamçı genelde saat yönünde olacak şekilde tek bir şekilde dönebilmektedir. Anaerobik koşullar altında bakterinin rotasyonunu yüzme sırasında duraksayarak sağladığı belirlenmiştir. Oryantasyonunu durduğu anlarda sağlar ve yüzerken hızı sürekli olarak değişkenlik gösterir. Ortalama yüzme hızı 35 μ m/s dir.

Fotosentetik üreme sırasında *R. sphaeroides* intrasitoplazmik bir membran sistemi sentezler. Membran sistemi fotosentezin birinci kısmı için gerekli olan bütün bileşenleri içerir. Bu bileşenler B875 α , B875 β ve B800-850 α ve B800-850 β 3 ışık toplayıcı kompleksten oluşur. Bu kompleksler ışık enerjisinin absorbe edilip enerjinin reaksiyon merkezine iletilmesinden sorumludur. CO₂ fiksasyonu için Kalvin döngüsü kullanılır. Kalvin döngüsünde yer alan iki enzim bu döngüde spesifiktir:

fosforibulokinaz ve ribuloz-1,5-bisfosfat karboksilaz/oksijenaz. Reaksiyon merkezi kompleksi H, M ve L olmak üzere 3 altbirimden oluşur. L ve M altbirimleri integral membran proteinleridir. Işık toplayıcısı (LH) ve reaksiyon merkezi (RC) pigment proteinlerinin kodlanmasında rol alan genler 2 farklı operonda yer almaktadır. LH1, RC-L ve RC-M polipeptidleri puf operonundaki genler tarafından kodlanırken, LH2 puc operonunda yer alan genler tarafından kodlanır. Her ne kadar ışık yoğunluğu bu genlerin regülasyonunda önemli bir rol olsa da asıl kontrol edici faktör oksijen yoğunluğudur. *R. sphaeroides* aerobik ortamlarda, oksijeni elektron alıcı olarak kullanarak kemotrofik olarak büyüeyebilen fakültatif anaerob bir organizmadır. Oksijen seviyesinin eşik değeri altına düşmesi durumunda fotosentetik pigmentler ve pigment bağlayıcı proteinlerin sentezi tetiklenir. *R. sphaeroides*; hidrojen gazı, polihidroksibutirat, B₁₂ vitamini, koenzim Q10, 5-aminolevulinik ve porfirin üretiminde kullanılan, endüstriyel ve tıbbi alanda önemli yere sahip bir organizmadır. Farklı stres koşullarında izlediği stratejiler nedeni ile biyoremediasyon uygulamalarında kullanılır. Ağır metal stresine maruz kaldığında, membran yapısında bulunan yağ asidi miktarını değiştirerek membranını sağlamlaştırma yoluna gider. Co²⁺ gibi divalent katyonları dış membranına bağlayabilmesi ağır metal direncine katkı sağlayan stratejilerden biridir. *R. sphaeroides*'in bu geniş metabolik repertuarı direnç metabolizmalarının anlaşılması açısından bilim çevresinde büyük bir araştırma alanı oluşturmıştır.

Nevrit ve anemi tedavisinde kullanılan B₁₂ vitamininin sentezi *R. sphaeroides* tarafından aerobik ve anaerobik olarak sağlanır. *R. sphaeroides* P47 suşu karanlık aerobik şartlar altında 75 µg/g kuru ağırlığında B₁₂ vitamini sentezleme kapasitesine sahip iken aynı suş, aydınlık anaerobik şartlar altında 87 µg/g kuru ağırlığında B₁₂ vitamini sentezleyebilmektedir.

Serbest radikallerin nötralizasyonunda kullanılan koenzim Q10 antioksidanı *R. sphaeroides* tarafından aydınlık anaerobik koşullar altında, karanlık ve aerobik koşullar altında sentezleyebildiğinin 2 katı kadar sentezlenir.

Tetrapirel biyosentezinde ara ürün olarak sentezlenen 5-aminolevulinik asit (ALA) *R. sphaeroides* tarafından levulinik asit kullanılarak sentezlenir.

Bu çalışmada endüstriyel ve tıbbi alanda bu kadar geniş bir kullanım alanına sahip olan *R. sphaeroides*'in Co²⁺ ağır metale karşı olan direncinin geliştirilmesi amaçlanmıştır. Yüksek kobalt konsantrasyonlarını tolere eden suş, evrimsel mühendislik yöntemi ile elde edilmiştir. Evrimsel mühendislik yaklaşımı bakterinin artan kobalt konsantrasyonlarında üretilmesini gerektirdiğinden ilk adım olarak seleksiyon sürecinin başlangıç stres konsantrasyonu belirlenmiştir. Evrimsel mühendislik yaklaşımı, çalışılan organizmanın metabolizması hakkında geniş bilgi sahibi olunmasını gerektirmez. Başlangıç konsantrasyonu, referans *R. sphaeroides* R-26' nın 11 farklı kobalt konsantrasyonunda büyütülerek taranması ile bulundu. Tarama sonuçlarına göre popülasyonların eldesi 0,1 mM kobalt konsantrasyonu içeren besiyeri ortamında başlatıldı. Kobalt konsantrasyonu 0,1 mM' dan 15 mM'a kademeli olarak arttırılarak 64 nesil elde edildi. Sağkalım yüzdeleri her 24 saatte bir kontrol ve stres kültürlerinin OD₅₃₅ değerleri ölçülerek hesaplandı. OD₅₃₅ ölçümleri yapılırken, diğer yandan kültürler LB katı besi yerlerine ekilerek kontaminasyon kontrolü yapıldı. Katı besi yerlerinde üretilen hücreler morfolojik olarak analiz edildi. Karotenoid üretme yeteneğine sahip ve karakteristik kırmızı renk veren hücrelerin varlığı, kontaminasyon olmadığının göstergesi olarak kullanıldı. Sağkalım yüzdesi 0.2 değerine düştüğünde popülasyon eldesi sonlandırıldı. Son popülasyon olarak 64. nesil belirlendi.

Popölasyonun eldesinin ardından 64. nesil ve referans költürüne ait hücrelerin gelişme eğrileri, kobalt içeren ve içermeyen olmak üzere iki farklı ortamda elde edildi. Ortamda kobaltın olmadığı durumlarda referans költürü ve 64. nesil benzer üreme özelliğı göstermiştir. Diğer yandan 4 mM kobalt içeren besiyeri ortamlarında üretilen költürlerden elde edilen gelişme eğrileri önemli oranda farklılık göstermiştir. 64. nesile ait hücrelerin 4 mM kobalt konsantrasyonu varlığında gösterdikleri üreme stressiz besi ortamında gösterdikleri üreme ile benzerlik gösterirken, referans költüründeki hücrelerin üremesinin önemli ölçüde inhibe edildiğı gözlemlenmiştir. Bu sonuçlar, 64. neslin kobalt stres direncinin geliştirilebildiğinin göstergesidir.

1. INTRODUCTION

1.1 General Information About *Rhodobacter sphaeroides*

Rhodobacter sphaeroides is a phototrophic bacterium that is able to produce energy through photosynthesis. It has a diverse metabolism. Besides being capable of growing photosynthetically, it can grow heterotrophically using either aerobic or anaerobic respiration. *R. sphaeroides* is unable to use sulphur as electron acceptor, but uses hydrogen as a source of reduction power and nitrogen as a source of organic nitrogen. It has a single subpolar flagellum, which enables its movement. Kaplan et al. (2006) stated that *R. sphaeroides*' closest relative is *Silicibacter pomeroyi*, a member of the marine *Roseobacter* clade. They proposed a scenario to explain the evolutionary mechanism of *R. sphaeroides*. According to this scenario, in the beginning the earth was covered with ocean. As the land started rising and long periods of rainfall took place, the lakes reduced their salinity. This gave rise to the formation of today's freshwater bodies of water. It is thought that the ability of *R. sphaeroides* to photosynthesize has developed before the fall in the sea level. This would explain the global distribution of similar freshwater non-sulphur photosynthetic bacteria.

8300 proteins of *R. sphaeroides* have been identified. It contains a flagellum both structurally and functionally similar to that of *E. coli*. It has two different systems: a sub-polar flagellum and multiple –polar flagella (Del Campo *et al*, 2007).

1.2 Genetic Properties

R. sphaeroides consists of two circular chromosomes (C1, 3.188.631 bp and C2, 943.022 bp) and five endogenous circular plasmids giving a total genome size of 4400 kb. Four of the plasmids are approximately 100 kb and the smallest one is around 42 kb (Suwnato and Kaplan, 1989), (Figure 1.1). Puf operon contains structural gene information for light harvesting and reaction centre protein complexes. These protein complexes are present in a fixed ratio within the photosynthetic intracytoplasmic membrane (Kaplan *et al.*, 1988). *Puh* operons are involved in the RC assembly (Zheng *et al.*, 2011).

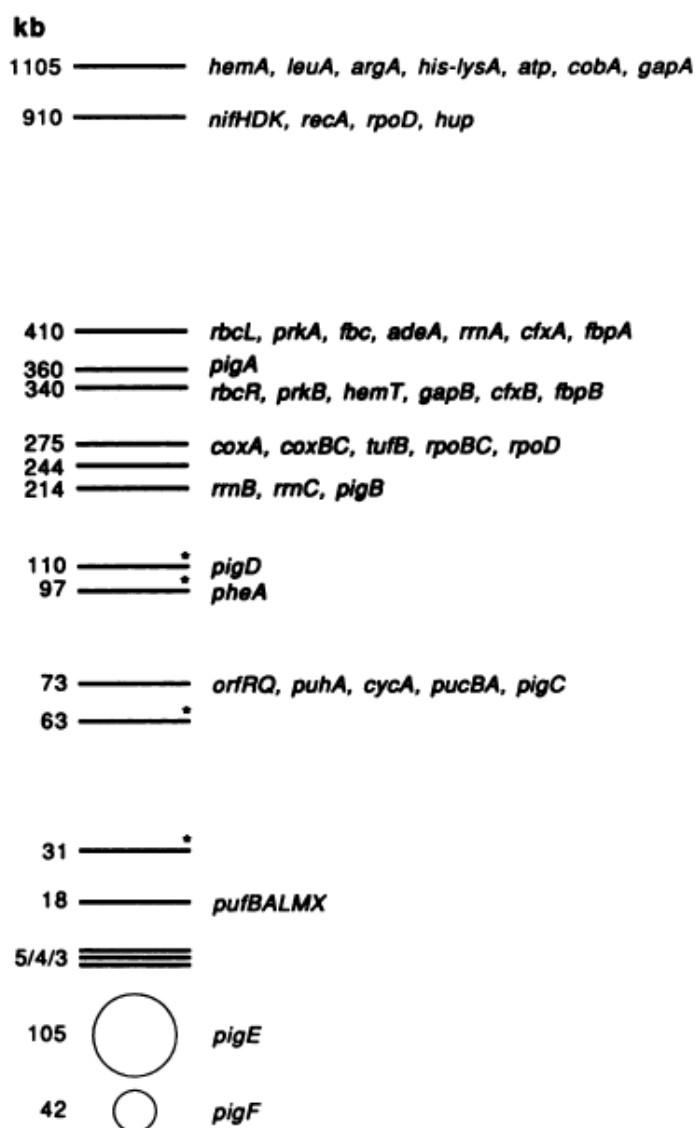


Figure 1.1 : Localization of genes and operons in *R. sphaeroides* (Suwnato and Kaplan, 1989).

C1 and C2 contain 3106 and 874 open reading frames, respectively. *R. sphaeroides* contains a great amount of exact DNA sequence duplications (Table1.1). Gene duplication generates the genetic diversity that allows *R. sphaeroides* to widen its spectrum of metabolic capabilities (Choudhary et al., 2004).

Table 1.1 : Large duplication regions in *Rhodobacter sphaeroides* (Choudhary et al., 2004).

Duplicated region	Coordinates on chromosome	Length (kb)
CIA1 (<i>rrnA</i>)	0-5350	5.35
CIB1	222609-253700	31.1
CIB2	737289-762734	25.45
CIIA2 (<i>rrnC</i>)	0-5350	5.35
CIIA3 (<i>rrnB</i>)	33674-39022	5.35
CIIB1	645366-673966	28.6
CIIB2	922523-891191 reverse	34.8

1.3 Chemotaxis

R. sphaeroides has a laterally arising single flagellum. It is able to rotate only in one direction which usually is clockwise. Under anaerobic conditions, *R. sphaeroides* was observed to swim and stop, rather than swimming and tumbling. It re-orientes during a stop and while swimming it shows a big variation in its swimming speed. The mean swimming speed is 35 $\mu\text{m/s}$ whereas *E. coli*'s is 20 $\mu\text{m/s}$. The genetic organization of the chemosensory genes in *R. sphaeroides* shows considerable similarity with the other group members of α -subgroup. At least two copies of *cheY* gene is found to be common in *R. sphaeroides*, *S. smeliloti* and *E. coli*. *R. sphaeroides* differs in possessing a functional set of the chemotaxis genes (*cheA*, *cheW* and *cheR*) and a third copy of *cheY* gene (Armitage and Schmitt, 1997). *CheA*, *cheW* and *cheY* are essential for taxis to PTS sugars, oxygen and MCP-dependent chemoeffectors. *R. sphaeroides* does not show receptor-dependent chemotaxis. It can grow as a phototroph anaerobically in the light, or as a heterotroph, aerobically in the dark. *R. sphaeroides* shows response to a wide range of stimuli. The predominant chemoattractants are weak organic acids such as propionate. Responses to certain stimuli depend on the conditions under which cells have been grown. When cells are grown photoheterotrophically in the light, they show a positive response to increasing light intensity but are repelled by high oxygen concentrations. Cells show positive response to oxygen when they are incubated in the dark. This shows that components of the chemosensory system are under environmental control (Porter et al., 2008). The chemotaxis signalling network in *R. sphaeroides* is shown in Figure1.2.

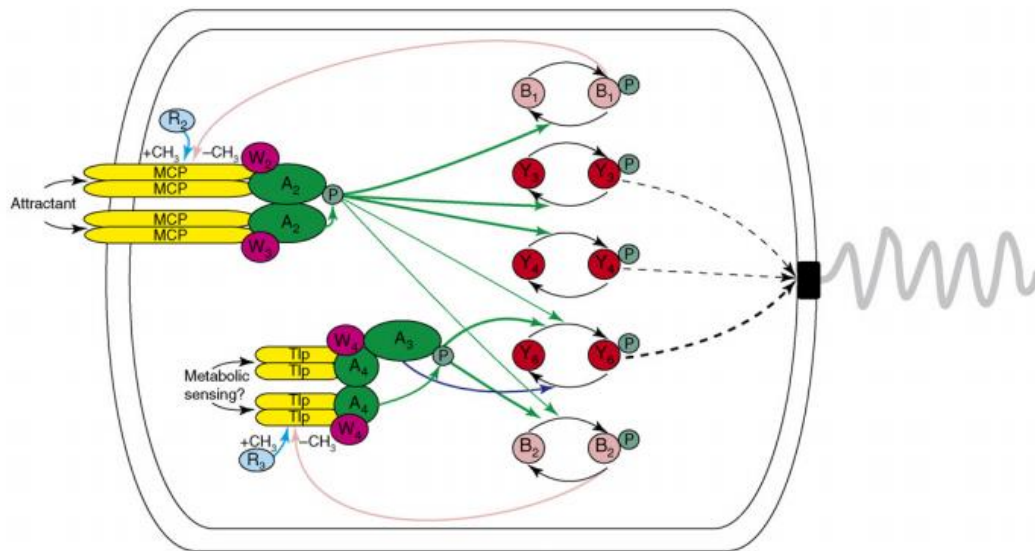


Figure 1.2 : Chemotaxis signalling network in *R. sphaeroides* (Porter et al., 2008).

1.4 Photosynthesis

During photosynthetic growth, *R. sphaeroides* synthesizes an intracytoplasmic membrane system. This membrane system contains all the components that are necessary for primary photosynthesis. These components consist of B875 α , B875 β and the B800-850 α and B800-B850 β light harvesting complexes. These complexes take a role in absorbing light energy and transferring this energy to the reaction centre. To fix carbon dioxide *R. sphaeroides* uses Calvin cycle. Two of the enzymes synthesized in this cycle are unique for Calvin cycle: phosphoribulokinase and ribulose-1,5-bisphosphate carboxylase/oxygenase. *R. sphaeroides* possesses 3 distinct pigment-protein complexes that take role in transduction of light energy into chemical energy. Two of these complexes are light harvesting (LH) complexes LH1 and LH2, also known as B875 and B800-850 antenna complexes. The Reaction Centre (RC) complex is composed of three subunits: H, M and L. The L and M subunits are integral membrane proteins. Genes involved in encoding of LH and RC pigment protein complexes are clustered on two different operons. The genes encoding LH1 polypeptides (pufA, pufB), RC-L and RC-M (puf-L, pufM) compose the puf operon. On the other hand, genes encoding LH2 polypeptides constitute the puc operon. Light intensity has a key role in the regulation of the size of the photosynthetic apparatus, but the major controlling factor is oxygen. *R. sphaeroides* is a facultative anaerobe that is able to grow chemotrophically, using oxygen as electron acceptor under oxic conditions. The formation of photosynthetic pigments and pigment-binding proteins is

triggered by lowering the oxygen below a threshold. Biosynthesis of Bch1 and pigment binding proteins is coordinated with the decrease of oxygen level. The regulation of *puf* and *puc* operons is enabled by transcriptional and post-transcriptional control (McEwan A.G., 1994). The photosynthetic apparatus consists of 3 compartments: light-harvesting complex (LHC), reaction centre and cytochrome (cyt) *bc*₁ complex. LHCs bind carotenoid and bacteriochlorophyll molecules non-covalently. They are responsible for collecting the light energy. LH2 is modulated by light intensity and oxygen partial pressure. LH1 forms RC-LH1 complexes with RC. LHC absorbs a photon and at the RC the electron is transferred from a bacteriochlorophyll dimer to a bacteriopheophytin and subsequently to the primary quanine (Q_A). Next, the electron is transferred from Q_A to Q_B (a second ubiquinone molecule). During this step, Q_B forms a RC-bound semiquinone. This cycle is repeated giving a doubly reduced Q_B. The doubly reduced Q_B receives 2 protons from the cytoplasmic space. After that step, Q_B is released from RC to the quinone pool in the form of quinol (QH₂). The quinol is oxidized by cyt *c*₂, releasing two protons to the periplasmic space. The electron transport cycle is completed by the periplasmic space. The electron transport cycle is completed by the reduction of the photo-oxidized primary electron donor (Vermeglio and Joliot, 1999), (Figure 1.3).

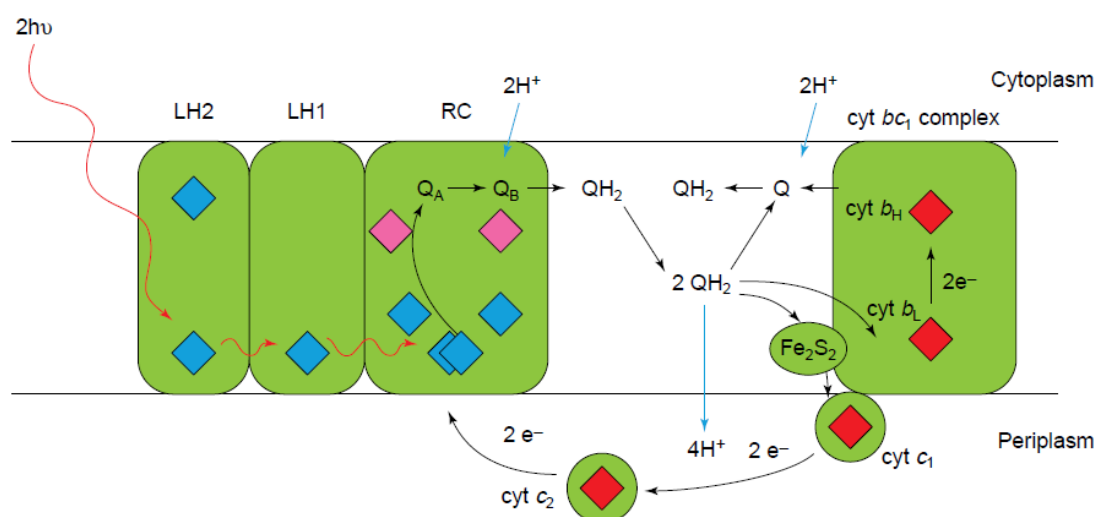


Figure 1.3 : Schematic representation of the photo-induced cyclic electron transfer (Vermeglio and Joliot, 1999).

1.5 Metabolic Strategies of *R. sphaeroides*

R. sphaeroides is a metabolically diverse bacterium. The rich repertoire of energy generation modes puts this organism in the centre of regulatory network studies. The non-pigmented or lightly pigmented *Escherichia coli*-like cells are transformed to pigmented cells with intracytoplasmic membranes (ICMs) that contain protein-pigment photosynthetic complexes when O_2 tension is decreased. Genes responsive to O_2 constitute the largest cluster of co-regulated genes (approximately 600 ORFs). This proves that O_2 plays a major role in controlling *R. sphaeroides* metabolism and physiology (Figure 1.4). Many of the O_2 -responsive genes encode proteins that are crucial for photosynthesis. These include light energy harvesting proteins, structural proteins of photosynthetic complexes and enzymes involved in the biosynthesis of carotenoid and bacteriochlorophyll clustered in photosynthesis gene cluster.

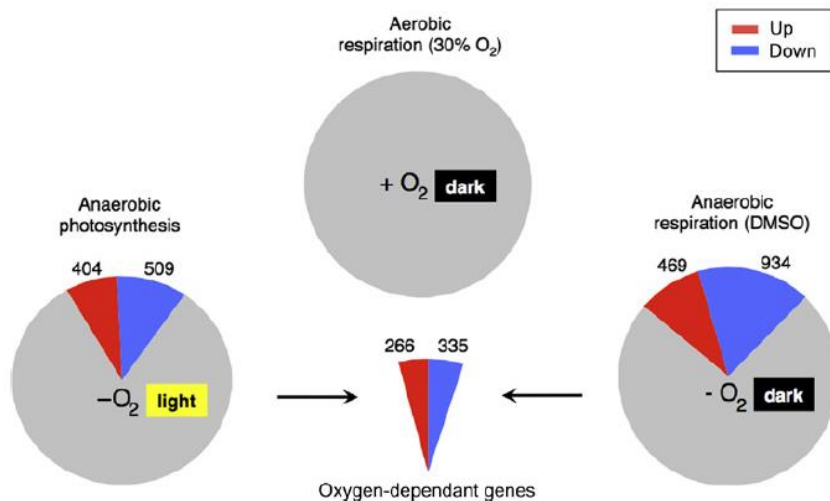


Figure 1.4 : Transcriptome profiles of *R. sphaeroides* (Gomelsky et al., 2013).

Succinate dehydrogenase (sdh) which is the enzyme that catalyses the oxidation of succinate to fumarate with reduction of ubiquinone to ubiquinol, is regulated at the transcriptional level. The genes of this enzyme are downregulated under photosynthetic conditions, consequently. Decreased *SDH* gene expression may be explained by a lower utilisation of succinate. There are two factors that explain the downgrade: 1- The energy produced by photosynthesis decreases the need for NADH generated from succinate oxidation. 2- Additional source of carbon is rendered by CO_2 fixation during photosynthesis. The upregulation of the two genes involved in CO_2 fixation under photosynthetic conditions supports this possibility. Two of the major cyt c gene products take role in the transfer of electrons from the bc1 complex. Both

of the cytochromes transfer electrons to the cyt c oxidases but cyt c2 is the only one transferring the electrons to the photosynthetic RC. This explains the upregulation of cyt c2 gene and the downregulation of cyt c(y) gene under anoxic condition. An experiment that involved profiling the transcriptome of *Rhodobacter sphaeroides* cultures under different concentrations of O₂ (from 30 to 0.5% O₂) revealed that the expression of PS genes increased gradually as O₂ tension decreased (Huang & Kaplan, 1973). Roh *et al.*, (2004) examined the role of light in anaerobic photosynthetic cultures indicating that the cell growth rate decreased under low light intensity (3W/m²) significantly, whereas mRNA levels of most of the PS genes differed slightly. These results suggest that post-transcriptional regulations play a major role in controlling light-dependent abundance of photosynthetic complexes under aerobic conditions. On the other hand, it is stated that light plays a very important role in gene regulation under aerobic conditions (Gomelsky *et al.*, 2013).

1.6 Metabolic Regulation

Having a wide range of metabolic versatility, *R. sphaeroides*' metabolic network consists of Calvin-Benson-Bassham cycle, Embden-Meyerhof-Parnas pathway, Entner-Doudoroff Pathway, Pentose phosphate pathway and Tricarboxylic acid cycle (Tao *et al.*, 2012). Three regulatory systems are involved in the regulation of PS genes in response to different oxygen levels: 1- The PrrBA two subunit activation system 2- The PpsR repressor, 3-FnrL, anaerobic regulator. It is stated that cbb₃ cytochrome c oxidase and the Q pool act like redoxmeters. In response to oxygen availability, they measure oxygen levels and create signals to PrrBA system and PpSR repressor. FnrL (fumarate nitrate reduction) activates many genes involved in anaerobic energy generation in order to regulate the transition from an aerobic growth to anaerobic growth. Prp (photosynthetic response regulator) system consists of two components: PrrB sensory kinase located in the membrane and its cognate PrrA response regulator. This system activates the photosynthetic genes under anaerobic conditions. PrrBA system also regulates genes involved in CO₂, N₂ and H₂ utilization. PpSR is a redox responding protein with a mass of 54 kDa. It is a product of the PS gene cluster. It acts as a repressor under aerobic conditions and under high-light photosynthetic conditions (Oh and Kaplan, 2004), (Figure 1.5).

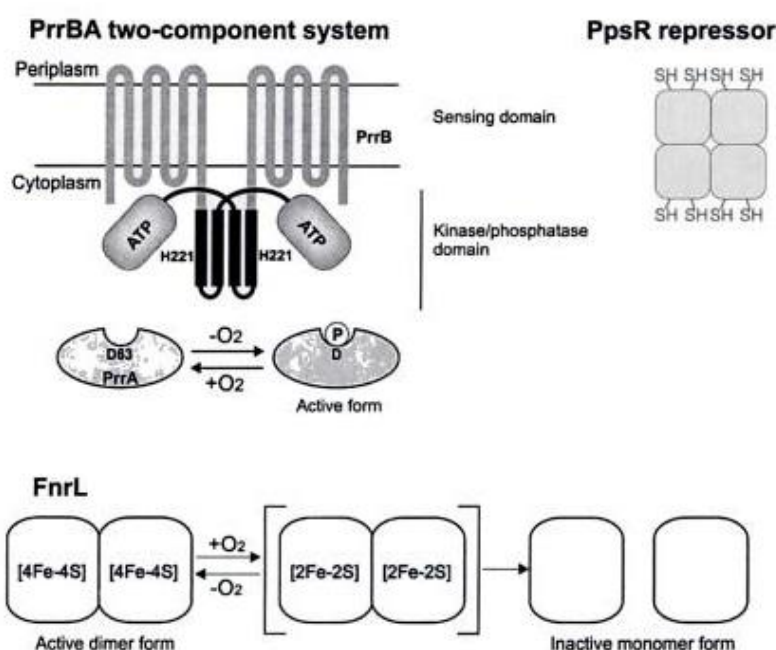


Figure 1.5 : Three major regulatory systems involved in metabolic regulation (Oh and Kaplan, 2004).

Table 1.2 : Different growth modes of RS (Koku et al., 2002).

Mode of growth	Carbon source	Energy source	Notes and relation to hydrogen production	Example strains
Photoheterotrophy	Organic carbon	Light	Preferred growth mode by PNS bacteria. Only mode resulting in hydrogen production.	O.U. 001
Photoautotrophy	Inorganic carbon (CO ₂)	Light	In the absence of organic carbon. Results in consumption of hydrogen.	2.4.1
Aerobic respiration (chemoheterotrophy)	Organic carbon	Organic carbon	In the presence of oxygen. Stops hydrogen production.	O.U. 001
Anaerobic respiration (chemoheterotrophy)	Organic carbon	Organic carbon	Under anaerobic, low light availability conditions. (Requires terminal electron acceptor other than O ₂ , such as nitrate). No hydrogen production. Marginal growth.	sp. denitrificans
Fermentation	Organic carbon	Organic carbon	Under anaerobic-dark conditions. No hydrogen production. Allows marginal growth.	ATCC 17023;

1.7 Respiratory Electron Transport System

R. sphaeroides carries out aerobic and anaerobic respiration (Table 1.2), and during respiration it uses both organic and inorganic compounds as electron donors. The respiratory electron transport chain (ETC) of *R. sphaeroides* consists of quinone pool, ubiquinol: cytochrome c oxidoreductase (the bc₁ complex) and a mobile cytochrome c₂. Under oxic conditions, type aa₃ and cbb₃ of cytochrome c oxidases and at least one functional quinol oxidase function as terminal oxidases. Cytochrome c₂ participates both in respiratory and photosynthetic ETC. Cytochrome c_y which is membrane anchored does not contribute to photosynthetic electron transfer. Bc₁ complex takes a central role between the transfer of electrons from Q-pool to cytochromes c₂ and c_y,

and the generation of a proton gradient across the cell membrane. Quinone is a lipid-soluble molecule that transfers electrons from NADH dehydrogenase and succinate dehydrogenase to the bc1 complex (Oh and Kaplan, 2004). Bc1 complex transfers electrons from the Q-pool to cytochrome c2 and cy while forming a proton gradient across the cell membrane. Bc1 consists of 4 subunits. Three of these subunits (cytochrome b, cytochrome c and the Rieske [2Fe-2S] protein) are encoded by the *fbcFBC* operon, whereas the smallest subunit is encoded by a separate *fbcQ* gene located on chromosome I and is responsible for the stability of the 3-subunit bc1 core complex. Cytochrome c2 and cy channel the electrons from bc1 complex to aa3 and cbb3 cytochrome c. The aa3 cytochrome c oxidase reduces dioxygen to one molecule of water and participates in the translocation of the protons across the membrane. Under aerobic respiration conditions, the major cytochrome c oxidase synthesized is aa3 cytochrome c oxidase. But when the oxygen tension is decreased, the biosynthesis of the cbb3 cytochrome c oxidase increases, whereas aa3 oxidase decreases (Oh and Kaplan, 2004).

1.8 H₂ Production

H₂ is produced as an obligatory product beside nitrogen fixation by nitrogenase (Tao *et al.*, 2012), (Figure 1.6). O₂ is the primary inhibitor of nitrogenase being able to destroy the enzyme irreversibly (Koku *et al.*, 2002). Nitrogenase synthesis is significantly induced by light. The enzyme contains molybdenum centre, therefore molybdenum is essential for the reaction (Koku *et al.*, 2002). The second enzyme involved in H₂ production is capable of both hydrogen production and hydrogen uptake. Nevertheless, the H₂ uptake activity of hydrogenase is more than hydrogen production activity. It is inhibited by carbon monoxide, oxygen and EDTA. Hydrogenase possesses nickel in its structure, therefore nickel is crucial for its activity (Koku *et al.*, 2002). H₂ production is inhibited by excess of nitrogen. NH₄⁺ inhibits the activity and gene expression of nitrogenase (Pandey *et al.*, 2012). Thus, nitrogen limitation should be ensured by a high C/N ratio such that bacteria can direct the excess energy and reducing power for the production of H₂ (Koku *et al.*, 2002).

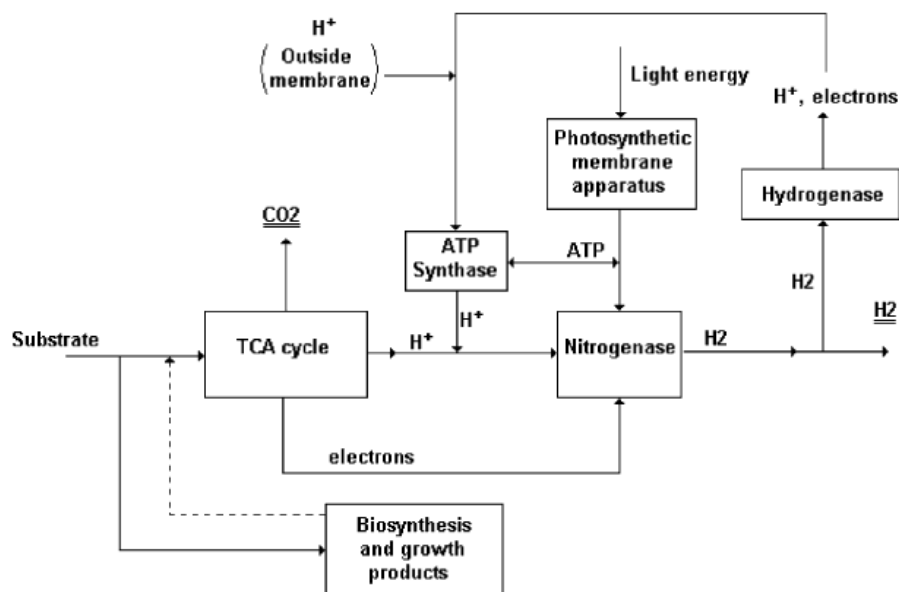


Figure 1.6 : Overall H_2 production scheme (Koku et al., 2002).

1.9 Metal Resistance

Metals which have a specific density of more than 5 g/cm^3 are commonly defined as heavy metals. These metals are not degradable and cannot be destroyed. Thus, bioremediation is used to immobilize the heavy metal pollutants in a non-bioavailable form or into less toxic forms. Metals fall into three main categories, according to their physiological properties: (i) essential and basically non-toxic, (ii) essential, but harmful at high concentrations, and (iii) toxic. Being an essential component of vitamin B_{12} , cobalt falls into the second category (Valls *et al.*, 2002), (Figure 1.7).

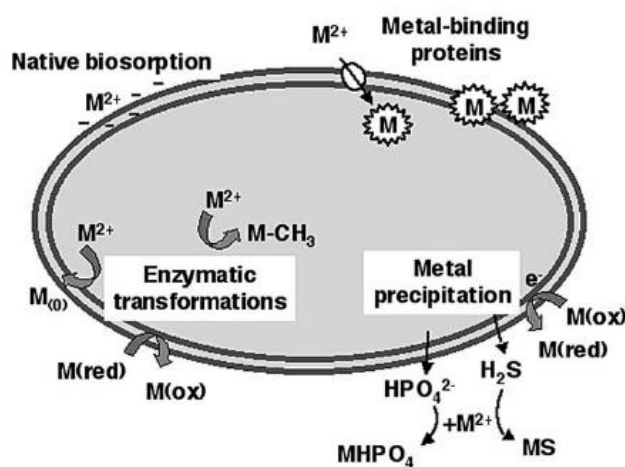


Figure 1.7 : Various means by which bacteria react to the presence of metals (Valls *et al.*, 2002)

Cobalt is a transition metal that can be found in two different oxidation states: Co^{2+} and Co^{3+} . It is present in the coring ring of enzyme B_{12} . Co^{2+} is known for its tendency to participate in Fenton-type reactions which involve special oxygen transfer properties that improve the use of hydrogen peroxide and make it potentially toxic at higher concentrations. Bacteria's response to Co^{2+} shows similarity with nickel and other divalent ions (Solioz *et al.*, 2011). Cobalt being able to increase the concentration of reactive oxygen species increases the oxidative stress in cells. Its toxicity is caused also by its tendency to bind the proteic $-\text{SH}$ groups of sensitive enzymes and subsequently inhibiting them or by replacing essential ions like magnesium and calcium (Pisani *et al.*, 2008). Divalent cations such as cobalt, copper, nickel and iron decreased the maximum growth rate and the population size at the stationary phase but did not affect the lag-phase duration. The growth curves obtained by addition of increasing amounts of Co^{2+} and Hg^{2+} showed different characteristics. While increase of Co^{2+} ions resulted in extended exponential phase, the increase of Hg^{2+} ions resulted in extended lag phase at a highly significant level (Giotta *et al.*, 2006), (Figure 1.8).

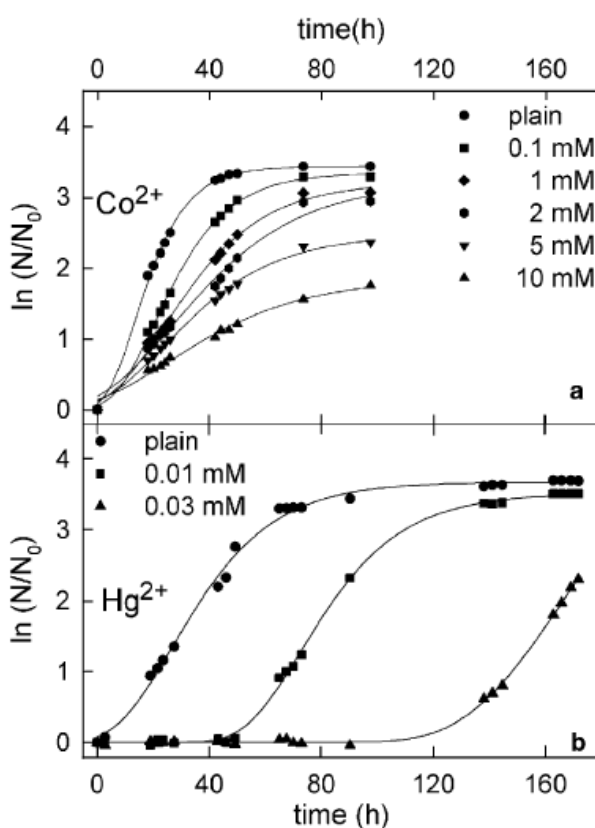


Figure 1.8 : Growth curves of *R. sphaeroides* R26.1 in the presence of Hg^{2+} and Co^{2+} (Giotta *et al.*, 2006).

The concentration-effect curves obtained helped identify a toxicity scale according to the $\mu_{\max}$ and EC_{50} values as follows: $Hg^{2+} > Cu^{2+} > CrO_4^{2+} \approx Ni^{2+} > Co^{2+} > Fe^{2+} > MoO_4^{2-}$. It was stated that as the sulphide solubility product K_{sp} of divalent cation decreased, the toxicity increased. The increased toxicity is a result of increment in the ability to bind to SH groups of sensitive enzymes (Giotta *et al.*, 2006). Co^{2+} tolerance of *R. sphaeroides* is reported to be particularly high when compared to that of other microorganisms (Giotta *et al.*, 2006). Co^{2+} has detrimental effect on the LH complexes. Bacteriochlorophyll biosynthesis drops (Giotta *et al.*, 2006). The effect of Ni^{2+} and Co^{2+} on maximum population size and pigment content is linked to the interference with magnesium transport and regulation (Giotta *et al.*, 2006). Kennedy *et al.* (1971) revealed in their study that a low level of Co^{2+} in the medium kills cells in the absence of Mg^{2+} , whereas the presence of Mg^{2+} can prevent this lethal effect of Co^{2+} . It was observed that when the medium contained 0.04 mM Mg^{2+} , the Co^{2+} concentration needed to inhibit the growth of *E. coli* was 0.1 mM, whereas when the Mg^{2+} concentration was increased to 1 mM, Co^{2+} concentration needed to inhibit the growth of the bacteria increased to 10 mM. These physiological responses of *E. coli* suggest an antagonistic relationship between Co^{2+} and Mg^{2+} uptake. The study supported the idea of involving a system responsible for both Co^{2+} and Mg^{2+} uptake. It was also stated that the uptake of Co^{2+} required energy such that cells incubated without energy source showed decreased Co^{2+} uptake. Also under conditions like low temperature, glucose, starvation or energy poison, Co^{2+} is not lethal as these conditions inhibit its uptake. In a similar study that was carried out by Webb and coworkers (2001), it was indicated that the energy-dependent uptake of Co^{2+} and Ni^{2+} was catalysed by the system normally responsible for Mg^{2+} , in *Aerobacter aegenes*. They supported this assumption by observing that Co-resistant *Aerobacter* variants were defective in the uptake of both Co^{2+} and Mg^{2+} (Nelson *et al.*, 1971). A recently published study by Italiano *et al.* (2014), indicated that *R. sphaeroides*' response to cobalt presence involved changes in the energetic metabolism. This statement was supported by reduced expression of subunits of an ABC sugar transporter operon on Co^{2+} -enriched medium. Among the genes involved in ABC transport system *rsp_1442* (coding for a substrate binding protein), *rsp_1443* and *rsp_1444* (coding for transporter permeases), *rsp_1446* (coding for sugarkinase) were upregulated in wild type *R. sphaeroides* exposed to Co^{2+} ions (Volpicella *et al.*, 2014). In 2008, Italiano *et al.* studied the effect of cobalt on *R. sphaeroides*. Experiments were carried out with carotenoidless mutants

that are able to grow photosynthetically under very low oxygen partial pressure. The study revealed that the increase of cobalt concentration in the growth medium results in the increase of antenna complexes that prevent the formation of dimeric units by causing a fraction of the constituent bacteriochlorophylls to loose (Pisani *et al.*, 2008). Aconitate hydratase is another enzyme spotted to be down regulated in the presence of Co^{2+} . This enzyme is essential for citric acid cycle as precursor for succinate biosynthesis. It reduces the biosynthesis of bacteriochlorophyll by reducing the succinyl-CoA synthesis. Succinyl-CoA is the precursor of 5-aminoleanilate, the starting substrate for bacteriochlorophyll synthesis. On the other hand, it was shown that the enzymes involved in DNA or protein degradation were overexpressed. The degradation products were used as the source of energy being used as substrates for gluconeogenesis enzymes. This explains the upregulation of the enzymes involved in glycogenosynthesis, glycogen mobilization and glycolysis in the presence of cobalt (Pisani *et al.*, 2008).

1.10 Evolutionary Engineering

Metabolic engineering plays an exceptional role in the improvement of industrially important microorganisms by intentional redirection of metabolic fluxes. This scientific discipline was first introduced by Jay Bailey in 1991. It was defined as “the improvement of cellular activities by manipulation of enzymatic, transport and regulatory functions of the cell with the use of recombinant DNA technology”. Later on, this definition was broadened by changing the “cellular activities” part to “product formation or cellular properties”, as reviewed by Nevoigt (2008). This change ensured that improvements as stress tolerance are included in the new definition. Indeed, Bailey redefined metabolic engineering as “the improvement of industrial organisms using modern genetic tools” (Bailey, 1999). Metabolic engineering is an interdisciplinary field that utilizes high-throughput technologies in functional genomics and bioinformatics. The general purpose in this field is to obtain improved or new proteins, improve chemical production by a host organism, extend substrate range for growth or modify cellular properties. Engineering approaches for microbial strain improvement are subdivided into two categories: Rational Metabolic Engineering and Inverse Metabolic Engineering. Rational metabolic engineering is the conventional approach which requires extensive information about the pathways, enzymes and their regulation. Improvement of the strains is carried out by engineering of enzymes,

transporters or regulatory proteins. Although this approach has been successful in a number of applications, many attempts failed resulting in less successful strains than predicted, or strains with undesirable side effects. Another disadvantage worth mentioning is that the features obtained under laboratory conditions were not transferrable to industrial conditions. These disadvantages may be overcome by a complete understanding of the complex global metabolic network. In 1996, Bailey introduced a new metabolic engineering approach to overcome the limitations of rational metabolic engineering. This approach was called “inverse metabolic engineering” (Bailey et al., 1996). In this “bottom-up” approach, the starting point is identification, isolation or calculation of desired phenotype. Next, the genetic basis or the particular environmental factors that confer that phenotype are determined. This step being the most challenging step involves high-throughput analytical technologies in functional genomics and proteomics. In the last step of inverse metabolic engineering, the identified genetic basis is transferred to a chosen, suitable industrial organism. Inverse metabolic engineering starting with the last step of rational metabolic engineering does not initially require extensive biochemical, genetic or regulatory information about the organism of interest (Çakar *et al.*, 2012). The identification, construction or calculation of a desired phenotype in the first step of inverse metabolic engineering could be obtained using evolutionary engineering. This approach involves mutagenesis and recombination of shuffling genes, pathways and whole cells followed by selection of cells with desired phenotype. Evolutionary engineering takes advantage of “natural design principles” and requires multiple cycles of random genetic perturbation followed by selection of phenotypically advantageous strains. Although evolutionary engineering was introduced to overcome the disadvantages of rational metabolic engineering, it has its own limitations. All methods of evolutionary engineering depend on suitable screening methods for the improved trait. Strain improvement starts with obtaining a pool of population with diverse genetic properties. This is achieved by exposing the organism to random mutagenesis using chemicals or UV light. The selection of desired variants is achieved by applying selection pressure throughout cultivations and identifying the fitter variants which can survive and grow better than the original cells. The random mutagenesis applied in the first step results in mutations spread over the entire genome. With no knowledge about the genetic alterations causing the desired phenotype it is

impossible to transfer the genetic information to the other strains. This issue can be overcome by using comparative transcriptome analysis (Çakar, 2009).

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Strain

In this study, the reference strain *Rhodobacter sphaeroides* R-25 was kindly provided by Dr. Massimo Trotta from University of Bari, Italy.

2.1.2 Growth Media

2.1.2.1 M27

R. sphaeroides was inoculated into liquid Medium 27 (M27) and Luria Broth agar plates throughout this study. The chemical composition of Medium 27 of the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) is listed in Table 2-1.

Table 2.1 : Chemical composition of Medium 27 of the DSMZ (Italiano et al., 2009).

Chemical	Concentration	Chemical	Concentration
<i>Macronutrients</i>	(mg l ⁻¹)	<i>Micronutrients</i>	(mg l ⁻¹)
KH ₂ PO ₄	500	ZnSO ₄ .7H ₂ O	100
MgSO ₄ .7H ₂ O	800	MnCl ₂ .4H ₂ O	30
NaCl	400	H ₃ BO ₃	300
NH ₄ Cl	400	CoCl ₂ .6H ₂ O	200
CaCl ₂ .2H ₂ O	50	CuCl ₂ .2H ₂ O	10
D,L malic acid	1500	NiCl ₂ .6H ₂ O	20
Yeast extract	2000	Na ₂ MoO ₄ .2H ₂ O	30
p-Amino benzoic	1		
Iron(II)citrate	18		
ddH ₂ O was added up to a final volume of 1000 mL.			
pH was adjusted to 6.8 using NaOH solution.			

2.1.2.2 Luria Broth (LB) Medium

Luria Broth (LB) plates were prepared using the ingredients listed in Table 2-2.

Table 2.2 : Ingredients of LB medium

Component	Amount (g)
Tryptone	10
Yeast Extract	5
NaCl	10
Agar	20
ddH ₂ O was added up to a final volume of 1000 mL.	

2.1.3 Laboratory Equipment

Laboratory equipment used in this study is listed in Table 2-3.

Table 2.3 : Laboratory Equipment

Equipment	Supplier	Country
Autoclave	Tommy SX700E	China
Benchtop Centrifuge	Beckman Coulter Allegra 25R	USA
Deep Freezer	-80 °C Sanyo Ultra Low MDT-	Japan
Desiccator	Finemech Bola-Star Vitrium	USA
Deep Freezer	-20 °C Arçelik 3011 NY	Turkey
Incubator	Nüve EN400-EN500	Turkey
Laminar Flow Hood	Biolab Faster BH-EU 2003	Italy
Light Microscope	Olympus CH30	Japan
Magnetic Stirrer	Labworld	Germany
Microbalance	Precisa 620C SCS	Switzerland
Microfuge	Eppendorf Microcentrifuge-5424	Germany
Micropipettes	Eppendorf	Germany
pH-Meter	Mettler Toledo MP220	Switzerland
Transilluminator	Vilber Lourmat	France
UV-Visible	Shimadzu UV-1700	Japan
Vortex Mixer	Heidolph	Germany

2.1.4 Chemicals, Buffers-Solutions and Software –Websites

Chemicals used in this study are listed in Table 2.4.

Table 2.4 : List of Chemicals

Chemicals	Supplier	Country
Agar	BD Difco™	USA
p-Amino benzoic acid	Fluka	USA
Ammonium chloride, NH ₄ Cl	Sigma ALDRICH	USA
Boric acid, H ₃ BO ₃	MERCK	Germany
Calcium chloride dihydrate, CaCl ₂ ·2H ₂ O	Carlo Erba	Italy
Cobalt chloride hexahydrate, CoCl ₂ ·6H ₂ O	Fluka	USA
Copper chloride dihydrate, CuCl ₂ ·2H ₂ O	Sigma ALDRICH	USA
Ethanol, C ₂ H ₆ O	J.T. Baker	The Netherlands
Glycerol, C ₃ H ₈ O ₃	Duchefa Bochemie	The Netherlands
Iron(II)citrate	Sigma ALDRICH	USA
Magnesium sulfate heptahydrate, MgSO ₄ ·7H ₂ O	MERCK	Germany
D,L malic acid	MERCK	Germany
Manganese chloride tetrahydrate, MnCl ₂ ·4H ₂ O	MERCK	Germany
Nickel chloride hexahydrate, NiCl ₂ ·6H ₂ O	MERCK	Germany
Sodium chloride, NaCl	MERCK	Germany
Sodium hydroxide, NaOH	MERCK	Germany
Sodium molybdate, Na ₂ MoO ₄ ·2H ₂ O	MERCK	Germany
Yeast Extract	MERCK	Germany
Zinc sulfate heptahydrate, ZnSO ₄ ·7H ₂ O	MERCK	Germany

Solutions and buffers used in this study are listed in Table 2.5.

Table 2.5 : Buffers and Solutions Used

Component	Amount
CoCl ₂ .6H ₂ O Stock Solution	500 mM Stock Solution
NiCl ₂ .6H ₂ O Stock Solution	100 mM Stock Solution
ZnCl ₂ .6H ₂ O Stock Solution	100 mM Stock Solution
CuSO ₄ .5H ₂ O Stock Solution	100 mM Stock Solution
NaCl Stock Solution	4 M Stock Solution
H ₃ BO ₃ Stock Solution	2 M Stock Solution
Glycerol	30% (v/v)
Ethanol	70% (v/v)
NaOH Stock Solution	10 M Stock Solution

2.1.5 Primers for Sequence Analysis

Primers designed for 16S rRNA analysis of *Rhodobacter sphaeroides* are listed in Table 2-6.

Table 2.6 : Primers Used in 16S rRNA analysis

	Sequence	Melting Temperature (T _m)
16sRNA_seq1_fw	5'-GCCTAACACATGCAAGTCGAGC-3'	56.7 °C
16sRNA_seq1_rev	5'-TTCCACTCACCTCTCTCGACC-3'	56.3 °C
16sRNA_seq2_fw	5'-TGACGGTACCACCAGAAGAAGC-3'	56.7 °C
16sRNA_seq2_rev	5'-ATCTCACGACACGAGCTGACG-3'	56.3 °C
16sRNA_seq3_fw	5'-ATTCTGAAGCAACGCGCAGAAC-3'	54.4 °C
16sRNA_seq3_rev	5'-ACGACTTCACCCCAGTCACTG-3'	56.3 °C

2.2 Methods

2.2.1 Preparation of Growth Medium

2.2.2 Screening

In order to determine the initial concentration of CoCl_2 for evolutionary engineering of *R. sphaeroides*, screening test was carried out. *R. sphaeroides* R-26 reference strain's pre-culture was prepared by inoculating 100 μL of the frozen stock culture to 15 mL of freshly prepared M27. The preculture was incubated overnight at 30°C in an incubator shaker at 250 rpm. Next day, the overnight culture cells were inoculated to an OD_{535} of 0.1 into M27 medium containing 2.5, 5, 6, 7, 8, 9, 10, 12.5, 15, 17.5, 20 mM cobalt concentration, as well as a control medium containing no cobalt. Cultures were incubated at 30°C in an incubator shaker. 19th, 25th, 42nd and 66th hour OD values were measured at 535 nm. Cells were spread on LB plates after each OD_{535} measurement to determine the viable cell counts. Cultures were diluted according to their OD_{535} values to adjust the concentration to 30-300 colonies per plate, in order to make the interpretation statistically meaningful.

2.2.3 Obtaining Co-resistant Strain through Evolutionary Engineering

Preculture was prepared as explained previously. Cells from this preculture were inoculated into 15 mL M27 medium with the determined initial Co^{2+} concentration along with a control medium with no cobalt. Cells were cultivated at 30°C and 250 rpm overnight. Optical density values were determined at 535 nm. Survival rate of the cultures was calculated by dividing OD_{535} of stress-treated cultures to that of the control group.

Selection was carried out by gradually increasing the Co^{2+} concentration in steps of 5%. Transition to the next generation was always performed by inoculating the previous stress culture at a starting OD_{535} of 0.1 into a control medium and a stress medium.

Before transferring to the fresh medium, cells were washed with M27 to remove Co^{2+} and maintain the controlled conditions. The washing procedure consisted of centrifuging the volume of cells corresponding to 0.1 OD_{535} in 15 mL volume for 5 minutes at 13,000 rpm. Cell pellets were resuspended in 1 mL of fresh M27 and centrifuged for 5 minutes at 13,000 rpm. This step helps suspend Co^{2+} from cell surface. Lastly, supernatant was removed and pellets were dissolved in 1 mL of M27.

The washed cultures were inoculated into a 50 mL test tube containing 14 mL M27 with the corresponding concentration of Co^{2+} .

2.2.4 Confirmation of the Strains

2.2.4.1 Spreading the Cultures to LB Plates

R. sphaeroides has a high risk of contamination requiring careful work under aseptic conditions and periodical confirmation. In order to ensure the transition of the cultures to the next generations without contamination, each generation was spread on LB plates, along with its control group. Colonies were confirmed as *R. sphaeroides*, based on their typical pigmentation and other morphological features. Cultures were serially diluted 10^{-4} fold and spread on LB plates. They were then incubated at 30°C, and cell growth was examined in 3-4 days.

2.2.4.2 PCR

As highly conserved regions, 16S rRNA genes were used to confirm the purity of *R. sphaeroides* culture during the experiments.

2.2.4.2.1 Template Preparation

DNA was extracted using Epicentre Biotechnologies (USA) MasterPure™ DNA Purification Kit (Cat. No. MCD85201). An overnight culture of 500 µL volume was centrifuged at 13000 rpm for 5 minutes. Supernatant was removed, leaving only 25 µL of it. After vortexing the pellets for 10 seconds, lysis solution consisting of 300 µL of Tissue and Cell Lysis Solution and 1 µL Proteinase K (50µg/µL) was added and mixed gently. The mixture was incubated at 65°C for 15 minutes. During this incubation, the sample was vortexed every 5 minutes. After cell lysis step, sample was cooled down to 37°C and 0.5 µL of RNase A (10µg/µL) was added. The mixture was incubated for 30 minutes at 37°C and for 3-5 minutes on ice. 300 µL of the cooled-down and lysed sample was mixed with 175 µL of MCP Protein Preparation Buffer. Ten seconds of vortexing and 10 minutes of centrifugation at 13000 rpm was performed subsequently. Supernatant was extracted and gently washed with 500 µL of isopropanol. Isopropanol was removed after 10 minutes of centrifugation at 13000 rpm. The pellet was washed twice with 70 % (v/v) ethanol by paying attention not to remove the pellet. The extracted DNA was resuspended in 35µL of TE buffer and stored at -20°C. Nano Drop

(Thermo SCIENTIFIC, USA) was used to determine the concentration of the extracted DNA.

2.2.4.2.2 Polymerase Chain Reaction

Polymerase chain reaction was carried out using three pairs of primers in order to obtain the whole 16S region. Reaction mixture was prepared by adding 2 μ L of dNTP (10mM), 2 μ L of 10X PCR buffer, 1 μ L of template (20ng/ μ L) 1 μ L of forward primer (2.5 μ M), 12.5 μ L DNA/RNase-free distilled water and 0.5 μ M of iTaq. The PCR cycles performed to amplify the 16S region are shown in Table 2-7 .

Table 2.7: PCR cycles performed to amplify 16S region of *R. sphaeroides*

Step	Temperature (°C)	Time	Number of Cycles
Initial denaturation	95	3 min	1
Denaturation	94	20 sec	34
Annealing	51	30 sec	
Extension	72	1 min	
Final Extension	72	5 min	1
Hold	5	∞	

2.2.4.2.3 Sequencing

Sequencing of the amplified 16S region was carried out by GENOMED AG Laboratories (Turkey).

2.2.4.3 Stock Preparation

Each of the obtained populations was given a number and stored at -80°C to be used for colony selection and further analysis. Stock preparation was carried out in triplets for each generation. The procedure consisted of centrifuging 3000 μ L of culture at 13000 rpm for 5 minutes, dissolving the pellet in 1 mL of fresh M27 to suspend CoCl₂ from the cells, centrifuging at 13000 rpm for another 5 minutes and dissolving the pellet in 30% (v/v) glycerol. Glycerol helps stabilize the frozen bacteria by preventing ice crystal formation which can have detrimental effects.

2.2.5 Growth Curve

R. sphaeroides reference strain and the 64th population pre-cultures were prepared by inoculating 100 µL of the stock to 15 mL of freshly prepared M27. After overnight incubation, pre-culture cells were inoculated with a starting OD₅₃₅ of 0.2 into 100 mL of pure M27 medium and 100 mL of M27 medium containing 4 mM CoCl₂. OD₅₃₅ measurements were carried out for 3 days to analyse the lag, exponential, stationary and death phase. Experiments were carried out using three separate replicates of the samples.

2.2.6 Cell Dry Weight Analysis

Two mL centrifuge tubes were dried in 80°C oven for 24 hours and subsequently kept in a desiccator for another 24 hours. The well-dried centrifuge tubes were weighed separately and labelled. Sampling for dry weight analysis was done at four different points of the cell growth. Two mL of culture was centrifuged for 5 minutes at 14000 rpm and dried at 80°C for 2 days. Later on, the dried tubes were weighed and cell dry weight was calculated by subtracting from previously obtained empty tube weights.

3. RESULTS

3.1 Determination of Initial Cobalt Concentration for Selection

Screening experiments were carried out to determine the initial CoCl_2 concentration to be applied during selection experiments of *Rhodobacter sphaeroides*. Survival rate was calculated by dividing the OD_{535} values obtained from the stress group to those of the control group. Percent survival rates at increasing CoCl_2 concentrations are plotted in Figure 3.1, for four different incubation times ranging between 17-66h.

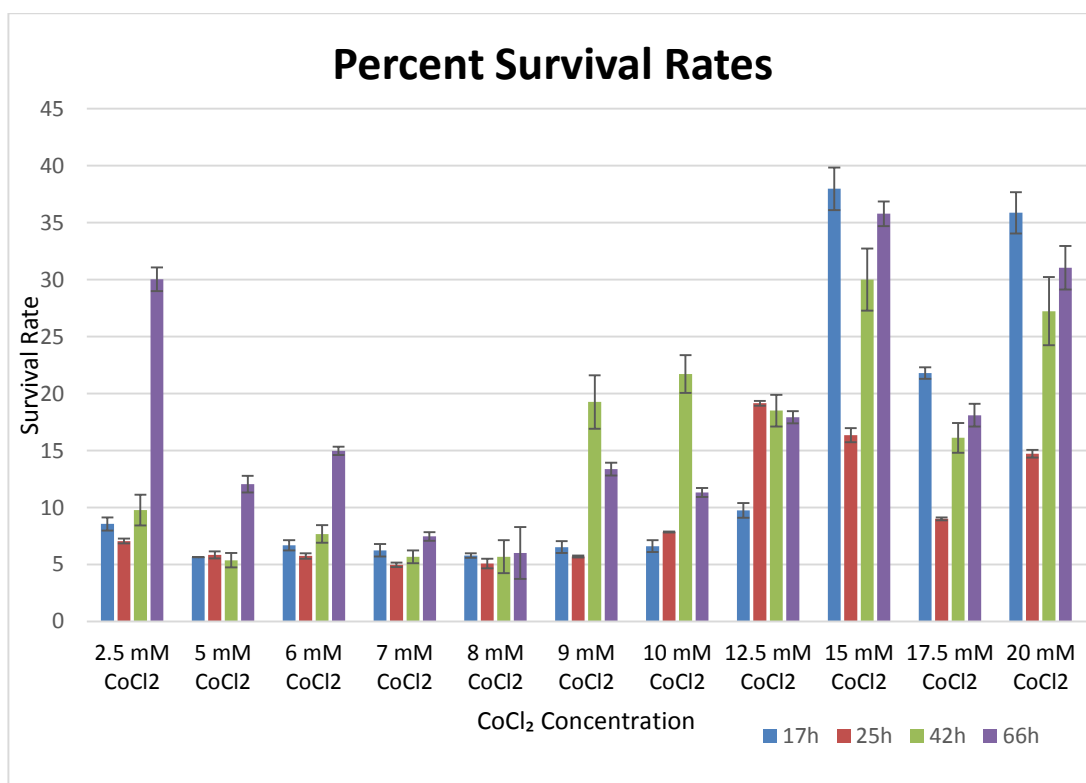


Figure 3.1 : Survival rates of *R. sphaeroides* at different Co concentrations.

Viable cell count analysis was performed to correlate the OD_{535} values with the actual cell numbers, and to omit the OD_{535} bias that may be caused by CoCl_2 . The obtained viable cell numbers per mL are plotted against time at different CoCl_2 concentrations in Figure 3.2.

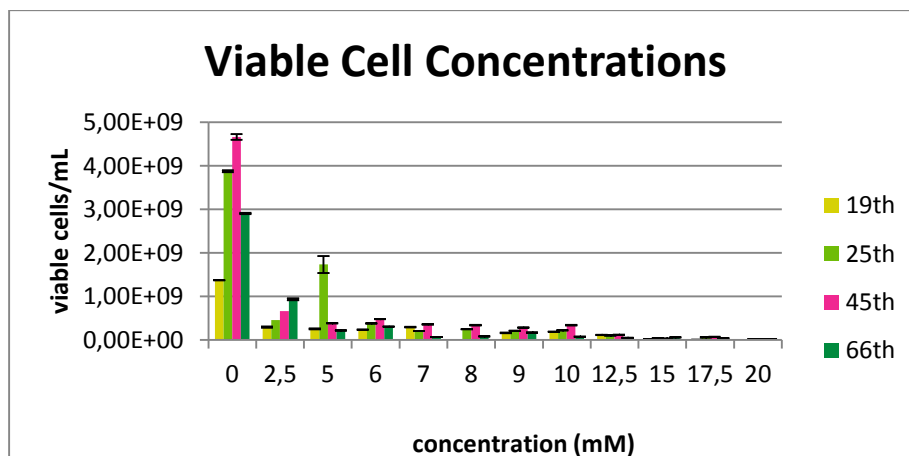


Figure 3.2 : Viable cell concentrations obtained from viable cell counts

Survival rates based on viable cell counts were calculated by dividing the number of viable cells in stress group to that in the control group. The obtained survival rates are plotted in Figure 3.3.

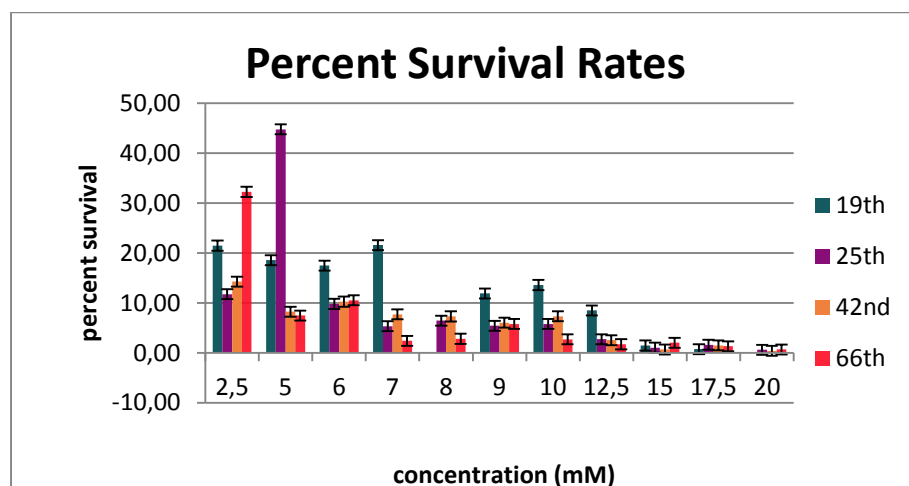


Figure 3.3 : Percent survival rates obtained from viable cell counts.

3.2 Selection Experiments

Based on the screening experiment results, it was decided to start selection with a low CoCl_2 concentration (0,1 mM) and gradually increase that concentration at each successive batch culture of selection. The CoCl_2 concentrations used at each passage of selection and the OD_{535} values obtained under control and the corresponding stress conditions are shown in Table 3.1. The 64th population that was obtained at 15 mM CoCl_2 stress was chosen as the final population of selection and the selection experiments were terminated at that level (Table 3.1).

Table 3.1 : OD₅₃₅ values obtained during selection

CoCl2 concentration	Population	Control Group					Stress Population				
		OD1	OD2	OD3	AVG	ST DEV	OD1	OD2	OD3	AVG	ST DEV
0.1 mM	1	2.69	2.86	2.67	2.74	0.11	3.13	3.15	3.17	3.15	0.02
0.15 mM	2	1.71	1.71	1.66	1.69	0.03	1.55	1.42	1.50	1.49	0.07
0.2 mM	3	2.12	2.11	2.14	2.12	0.01	2.43	2.63	2.44	2.50	0.11
0.25 mM	4	1.73	1.74	1.74	1.74	0.01	1.79	1.82	1.84	1.82	0.03
0.3 mM	5	1.95	1.96	1.21	1.71	0.43	1.74	1.72	1.78	1.75	0.03
0.35 mM	6	1.87	1.89	1.18	1.65	0.40	1.55	1.60	1.62	1.59	0.04
0.4 mM	7	1.76	1.74	1.83	1.78	0.05	1.25	1.31	1.32	1.29	0.04
0.45 mM	8	2.27	2.31	2.18	2.26	0.07	1.30	1.24	1.28	1.28	0.03
0.50 mM	9	1.32	1.35	1.29	1.32	0.03	2.10	2.00	2.05	2.05	0.05
0.55 mM	10	1.64	1.48	1.47	1.53	0.09	1.37	1.26	1.22	1.29	0.08
0.6 mM	11	1.21	1.26	1.26	1.24	0.03	1.35	1.35	1.45	1.38	0.06
0.65 mM	12	1.64	1.53	1.61	1.59	0.05	1.59	1.69	1.46	1.58	0.12
0.70 mM	13	2.44	2.58	2.49	2.51	0.07	1.60	1.76	1.64	1.66	0.08
0.8 mM	14	2.08	2.04	2.05	2.06	0.02	1.93	2.04	2.07	2.01	0.08
0.9 mM	15	2.11	2.15	1.97	2.08	0.09	1.98	1.95	1.88	1.93	0.05
1 mM	16	2.57	2.66	2.58	2.60	0.04	2.69	2.64	2.57	2.63	0.06
1.2 mM	17	3.31	3.18	3.04	3.18	0.13	1.86	1.71	1.71	1.76	0.08
1.4 mM	18	2.22	2.43	2.22	2.29	0.12	1.76	2.29	1.78	1.94	0.30
1.6 mM	19	2.46	2.20	2.27	2.31	0.13	1.24	1.18	1.55	1.32	0.20
1.8 mM	20	1.65	2.45	2.13	2.08	0.40	1.05	1.16	1.00	1.07	0.08
2 mM	21	2.58	2.22	2.14	2.31	0.23	1.67	1.71	1.68	1.68	0.02
2.2 mM	22	1.85	1.96	1.46	1.76	0.27	1.84	1.67	1.73	1.75	0.09
2.4 mM	23	1.90	1.94	1.90	1.91	0.02	1.47	1.60	1.64	1.57	0.09
2.6 mM	24	1.98	1.971	1.987	1.98	0.01	1.72	1.85	1.66	1.74	0.09
2.8 mM	25	2.36	2.32	2.42	2.37	0.05	1.80	2.06	2.19	2.01	0.20
3 mM	26	1.50	1.45	1.58	1.51	0.06	1.72	1.75	1.72	1.73	0.02
3.2 mM	27	2.58	2.79	2.59	2.65	0.12	1.45	1.45	1.49	1.46	0.02
3.4 mM	28	1.72	1.85	1.76	1.78	0.07	1.44	1.50	1.59	1.51	0.08
3.6 mM	29	2.17	2.27	2.22	2.22	0.05	2.47	2.47	2.52	2.49	0.03
3.8 mM	30	1.82	1.81	2.01	1.88	0.11	1.49	1.33	1.35	1.39	0.09
4 mM	31	2.08	2.07	2.24	2.13	0.10	1.30	1.32	1.44	1.35	0.08
4.2 mM	32	2.88	3.10	2.96	2.98	0.11	2.32	2.32	2.30	2.31	0.01
4.4 mM	33	2.55	2.42	2.44	2.47	0.07	1.51	1.56	1.60	1.56	0.04
4.6 mM	34	2.89	2.89	2.90	2.90	0.01	1.68	1.71	1.80	1.73	0.06
4.8 mM	35	1.90	1.95	1.89	1.91	0.03	0.88	0.90	1.04	0.94	0.08
5 mM	36	2.24	2.15	2.26	2.22	0.06	1.86	2.00	1.83	1.90	0.09
5.2 mM	37	2.24	2.32	2.23	2.26	0.05	1.73	1.57	1.63	1.64	0.08
5.4 mM	38	1.77	1.81	1.96	1.85	0.10	1.25	1.25	1.17	1.22	0.05
5.6 mM	39	2.61	2.40	2.51	2.51	0.11	1.68	1.81	1.64	1.71	0.09
5.8 mM	40	2.09	2.12	2.11	2.11	0.02	1.14	1.21	1.31	1.22	0.09
6 mM	41	1.40	1.40	1.38	1.39	0.01	1.26	1.18	1.23	1.22	0.04
6.2 mM	42	2.21	2.17	2.03	2.13	0.09	2.61	2.87	2.76	2.75	0.13
6.4 mM	43	1.98	1.94	1.93	1.95	0.03	1.13	1.23	1.25	1.21	0.06
6.6 mM	44	2.12	2.11	2.22	2.15	0.06	2.03	1.93	2.03	1.99	0.06
6.8 mM	45	2.24	2.16	2.07	2.15	0.09	1.87	1.79	1.52	1.73	0.19
7 mM	46	1.89	2.00	1.85	1.91	0.07	2.17	2.13	2.13	2.14	0.02
7.2 mM	47	1.57	1.48	1.59	1.55	0.06	1.23	1.10	1.16	1.16	0.07
7.4 mM	48	2.03	2.03	2.06	2.04	0.02	1.88	1.92	1.95	1.92	0.04
7.6 mM	49	3.29	3.14	3.32	3.25	0.10	2.49	2.47	2.52	2.49	0.02
8 mM	50	1.97	2.05	2.16	2.06	0.10	3.97	3.76	3.71	3.81	0.14
8.5 mM	51	1.57	1.71	1.52	1.60	0.10	0.66	0.63	0.66	0.65	0.02
9 mM	52	1.86	1.92	1.91	1.90	0.03	2.58	2.47	2.53	2.52	0.06
9.5 mM	53	1.79	1.57	1.68	1.68	0.11	0.55	0.66	0.62	0.61	0.06
10 mM	54	1.90	2.08	2.00	1.99	0.09	1.04	1.04	1.06	1.05	0.01
10.5 mM	55	1.98	1.97	2.14	2.03	0.10	2.50	2.49	2.55	2.51	0.04
11 mM	56	1.87	1.83	1.74	1.81	0.07	0.70	0.72	0.72	0.71	0.01
11.5 mM	57	1.45	1.50	1.56	1.50	0.05	1.13	1.17	1.14	1.14	0.02
12 mM	58	1.92	1.79	1.90	1.87	0.07	0.49	0.48	0.50	0.49	0.01
12.5 mM	59	1.80	1.57	1.64	1.67	0.12	0.83	0.81	0.79	0.81	0.02
13 mM	60	1.24	1.45	1.48	1.39	0.13	0.43	0.42	0.43	0.43	0.01
13.5 mM	61	1.79	1.71	1.74	1.75	0.04	0.48	0.50	0.48	0.48	0.01
14 mM	62	2.01	1.97	2.02	2.00	0.03	0.58	0.57	0.62	0.59	0.03
14.5 mM	63	1.64	1.64	1.68	1.65	0.03	0.47	0.55	0.44	0.49	0.06
15 mM	64	1.50	1.53	1.53	1.52	0.02	0.36	0.31	0.31	0.32	0.03

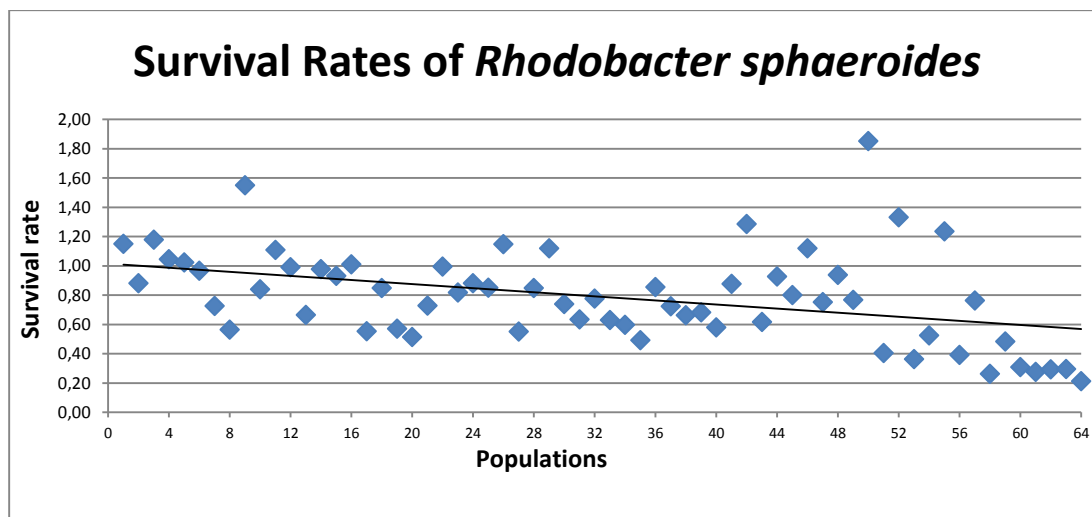


Figure 3.4 : Survival rate of each population obtained by selection.

3.3 Strain Confirmation

3.3.1 16S rRNA Sequencing

Sequencing results were aligned using web-based Basic Local Alignment Search Tool (BLAST). The obtained sequence was searched in the nucleotide database to analyse its similarity to *Rhodobacter sphaeroides*. According to the search results, the sequencing query showed 99% similarity with the 16S ribosomal RNA gene of *Rhodobacter sphaeroides* strain 2.4.1. A representative alignment result is shown in Figure 3.5.

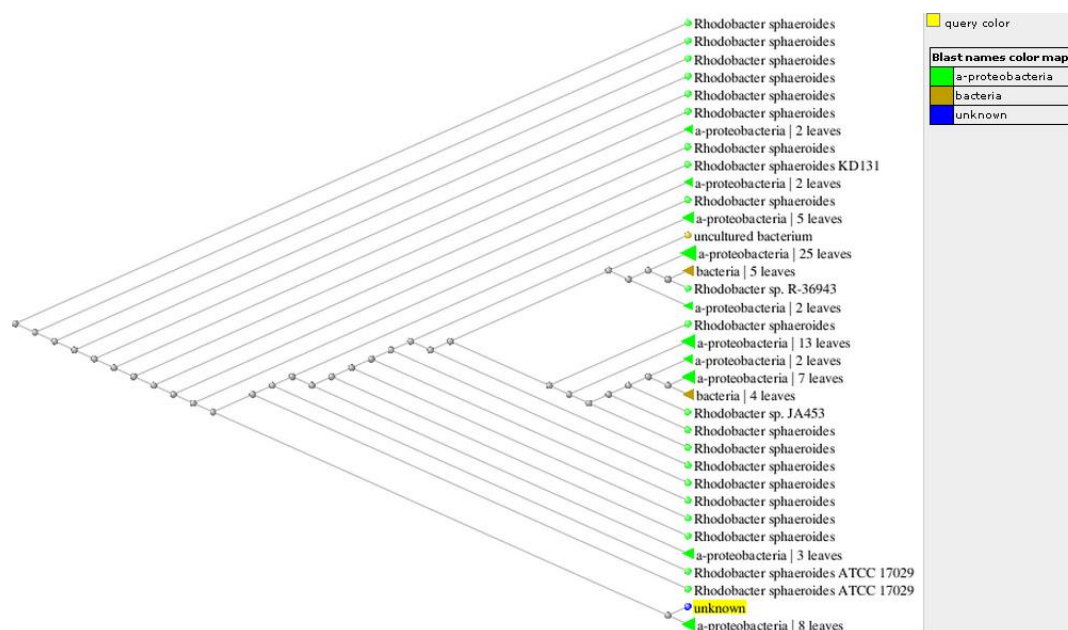


Figure 3.5 : 16S rRNA sequence alignment result of 49th population.

3.3.2 LB Plate Spreading

R. sphaeroides cultures spread on solid LB plates and incubated at 30°C for 3 days are shown in Figure 3.6. The typical red pigmentation is a simple indicator for the presence of *R. sphaeroides* colonies (Figure 3.6).

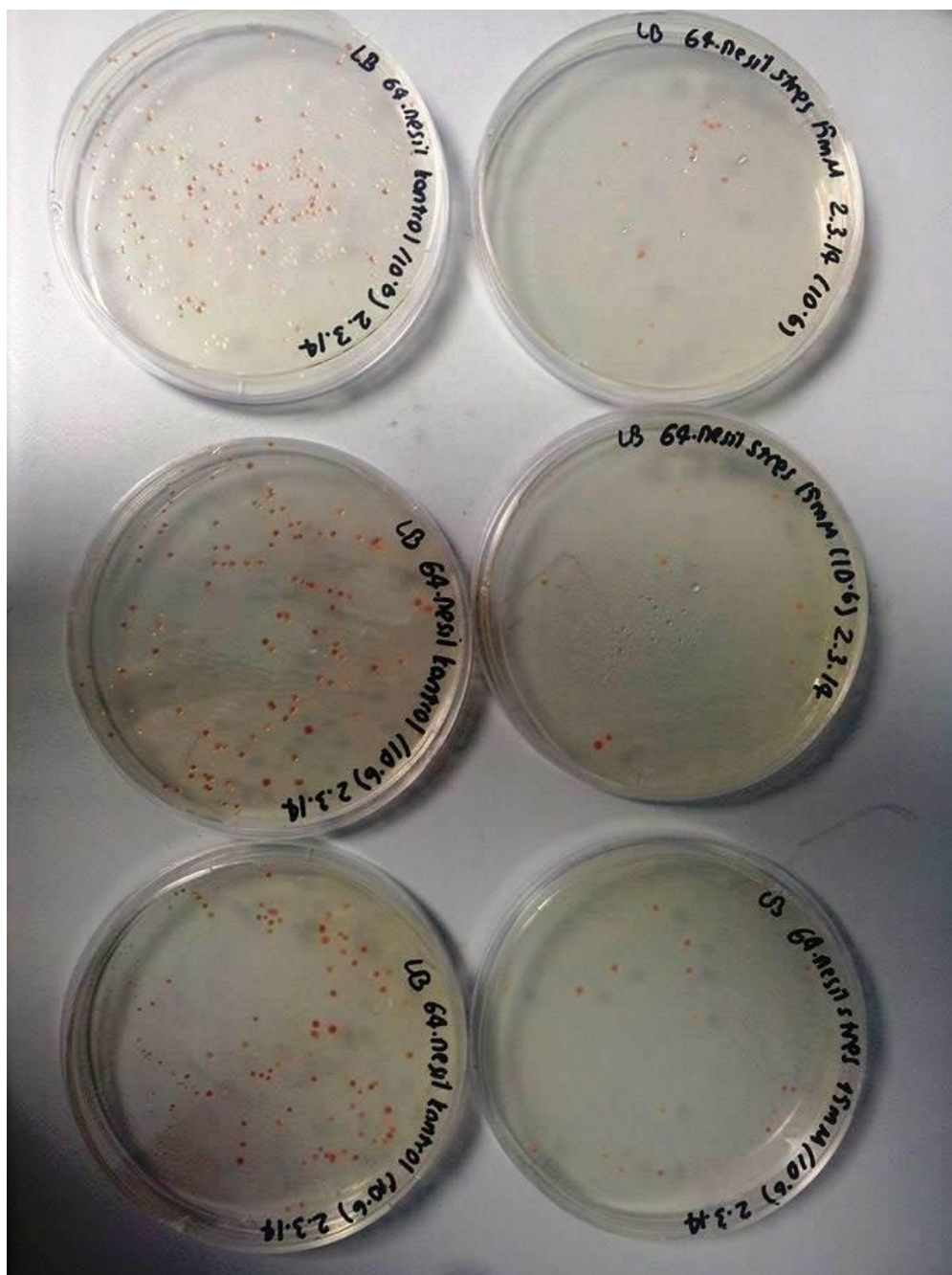


Figure 3.6 : The growth of *R. sphaeroides* in LB plates

3.3.3 Growth Curve

Growth curves of the reference strain and the 64th population were by obtained incubating the cultures in plain M27 medium and M27 medium containing 4 mM

cobalt. OD₅₃₅ values measured periodically are listed in Table 3-2. These values were plotted in Figure 3.6.

Table 3.2 : OD₅₃₅ values obtained during growth of the cultures

Reference strain	Hours	OD ₅₃₅ 1. repeat	OD ₅₃₅ 2. repeat	OD ₅₃₅ 3. repeat	Dilution Factor	Average	Std. Dev.
	2	0.192	0.1613	0.1897	2	0.362	0.0341
	3.5	0.2145	0.2174	0.1918	2	0.4158	0.0280
	5	0.3887	0.3972	0.3964	2	0.7882	0.0093
	7	0.7112	0.6831	0.6808	2	1.3834	0.0338
	9	0.5161	0.6045	0.5165	4	2.1828	0.2036
	11	0.6182	0.6626	0.6322	4	2.5500	0.0907
	13.5	0.528	0.5242	0.5795	5	2.7195	0.1544
	16	0.5933	0.6104	0.5616	5	2.944	0.1238
	21	0.3171	0.358	0.3619	10	3.4566	0.2481
	24.5	0.3058	0.3912	0.2886	10	3.2853	0.5494
	28	0.3271	0.3766	0.2933	10	3.3233	0.4189
	38.5	0.3049	0.3386	0.3295	10	3.2433	0.1743
	54.5	0.2974	0.4059	0.3009	10	3.3473	0.6165
	83	0.2753	0.3735	0.2898	10	3.1286	0.5300
64th population	Hours	OD ₅₃₅ 1. repeat	OD ₅₃₅ 2. repeat	OD ₅₃₅ 3. repeat	Dilution Factor	Average	STD.
	2	0.1949	0.1903	0.1897	2	0.3832	0.0056
	3.5	0.2222	0.2684	0.1963	2	0.4579	0.0730
	5	0.4268	0.4597	0.4146	2	0.8674	0.0466
	7	0.7281	0.8428	0.7362	2	1.5380	0.1280
	9	0.5299	0.5454	0.5326	4	2.1438	0.0331
	11	0.6428	0.6628	0.6437	4	2.5990	0.0451
	13.5	0.5237	0.5144	0.5795	5	2.696	0.1760
	16	0.546	0.5684	0.5421	5	2.7608	0.070
	21	0.3046	0.347	0.3015	10	3.177	0.2542
	24.5	0.3239	0.3243	0.3108	10	3.1966	0.0768
	28	0.2898	0.3019	0.2743	10	2.8866	0.1383
	38.5	0.2736	0.2891	0.2721	10	2.7826	0.0941
	54.5	0.2678	0.3319	0.2627	10	2.8746	0.3856
	83	0.2767	0.2439	0.2554	10	2.5866	0.1664
Reference strain-4mM Co	Hours	OD ₅₃₅ 1. repeat	OD ₅₃₅ 2. repeat	OD ₅₃₅ 3. repeat	Dilution Factor	Average	STD.
	2	0.0283	0.0065	0.1061	2	0.0939	0.1047
	3.5	0.1432	0.1525	0.1665	2	0.3081	0.0234
	5	0.2107	0.2083	0.2085	2	0.4183	0.0026
	7	0.2515	0.3027	0.2471	2	0.5342	0.0618
	9	0.1881	0.1578	0.1567	4	0.6701	0.0712
	11	0.1458	0.1453	0.1375	4	0.5714	0.0186
	13.5	0.1469	0.1427	0.1478	5	0.729	0.0136
	16	0.1805	0.1709	0.186	5	0.8956	0.0382
	21	0.1339	0.1307	0.1295	10	1.3136	0.0227
	24.5	0.1794	0.1041	0.0702	10	1.179	0.5589
	28	0.1965	0.1332	0.1278	10	1.525	0.382
	38.5	0.1897	0.1417	0.1796	10	1.7033	0.253
	54.5	0.2462	0.1731	0.259	10	2.261	0.4634
	83	0.2799	0.2135	0.2246	10	2.3933	0.3556
64th population-4mM Co	Hours	OD ₅₃₅ 1. repeat	OD ₅₃₅ 2. repeat	OD ₅₃₅ 3. repeat	Dilution Factor	Average	STD.
	2	0.0313	0.011	0.0032	2	0.0303	0.029
	3.5	0.2295	0.1935	0.2469	2	0.4466	0.0544
	5	0.3302	0.3134	0.3842	2	0.6852	0.0739
	7	0.4706	0.5446	0.5879	2	1.0687	0.1186
	9	0.3926	0.3832	0.4451	4	1.6278	0.1334
	11	0.5508	0.5161	0.4819	4	2.0651	0.1378
	13.5	0.4591	0.4366	0.5035	5	2.332	0.1702
	16	0.603	0.5765	0.5743	5	2.923	0.0798
	21	0.295	0.3453	0.3079	10	3.1606	0.2612
	24.5	0.2538	0.2562	0.2561	10	2.5536	0.0135
	28	0.3273	0.2913	0.2612	10	2.9326	0.3309
	38.5	0.2651	0.4042	0.293	10	3.2076	0.7358
	54.5	0.3005	0.2784	0.2758	10	2.849	0.1357
	83	0.2753	0.2542	0.2192	10	2.4956	0.2833

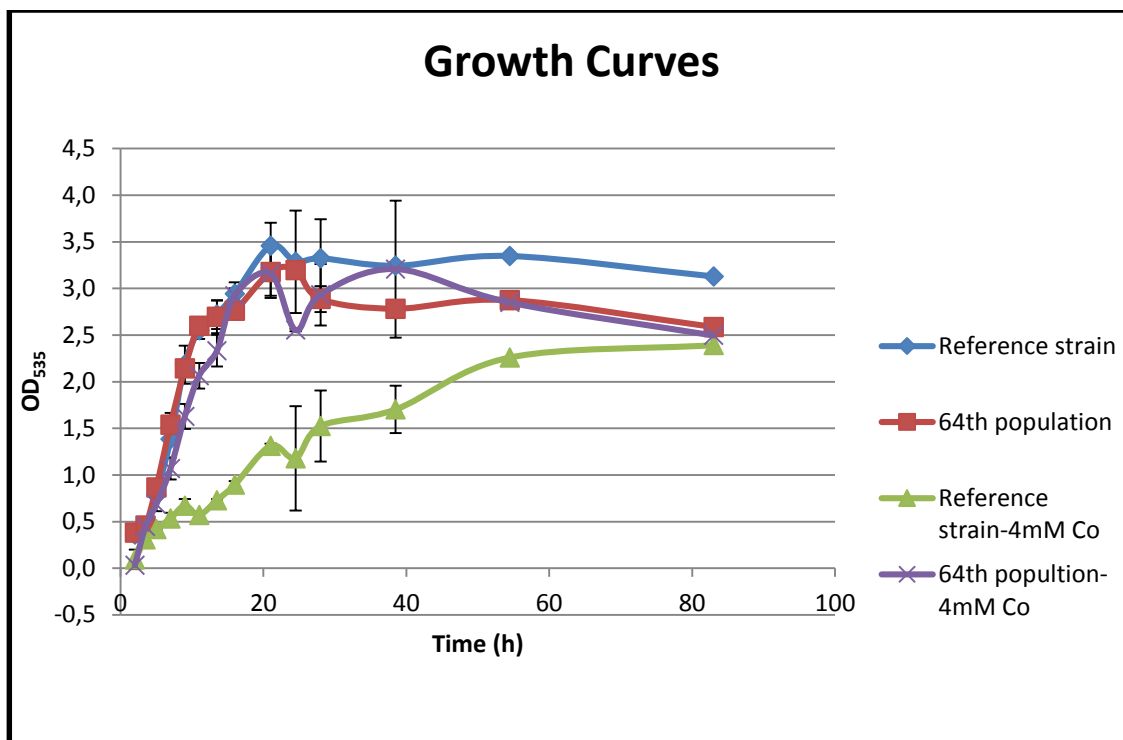


Figure 3.7 : Growth curves of RS and 64th population with and without cobalt.

4. DISCUSSION AND CONCLUSIONS

In this study, the reference strain of *Rhodobacter sphaeroides* was improved for CoCl_2 resistance. Initially, screening was carried out to determine the initial stress concentration of cobalt to be applied during selection experiments. Screening was performed for 11 different concentrations of CoCl_2 . OD_{535} values were obtained at 17th, 25th, 42nd and 66th hours of incubation. The increased concentration of cobalt resulted in false positive results which underlined the necessity of additional measurements. At the same time points of OD_{535} measurements, cultures were spread on LB plates to determine their viable cell numbers. The OD_{535} values were reliable until 8 mM of CoCl_2 concentration. Beyond 8 mM of cobalt, it was obvious that cobalt ions increased the optical density of the sample, possibly by forming a compound that absorbs light at 535 nm. Thus, the OD_{535} values showed an unexpected increase at 9, 10, 12.5, 15, 17.5 and 20 mM cobalt concentrations. However, survival rates that were calculated using viable cell counts showed a significant decrease with increasing concentrations of cobalt, as expected. According to the viable cell concentrations plot in Figure 3.2, the reference strain showed notable viability even at 10 mM concentration of cobalt. Among the four time points of measurements, the 25th hour gave the highest cell number for each concentration. These interpretations of viable cell count results indicated that 2.5 mM is a lethal dose for *R. sphaeroides*. In order to increase the stress resistance gradually, 0.1 mM CoCl_2 was chosen as the mild initial stress condition for selection experiments. CoCl_2 -resistant populations were obtained by increasing cobalt concentration gradually at each passage. Survival rates of each population were plotted in Figure 3.4 to monitor the resistance. The first 20 populations of selection had survival rates around 1 and higher, whereas the next 20 populations of selection (20-40) showed a slight decrease in their survival. After the 44th generation, which corresponds to a stress concentration of 6.6 mM cobalt, the survival rates fluctuated at high values. This could be a result caused by the excess cobalt concentration causing a bias in OD_{535} measurements. The last five generations -60, 61, 62, 63, 64- showed a drastic decrease in their survival rates. Selection was

terminated when the survival rate reached the value of 0.2. Strain confirmation was performed in two different ways: spreading each generation on LB plates to observe the typical red pigmentation, and sequencing 16S rRNA regions every 20 generations. In Figure 3.6, colonies of 64th population are shown. Cells have the characteristic red colour for *R. sphaeroides* originating from carotenoid biosynthesis, implying that the culture has not been contaminated. 16S rRNA sequencing data was aligned using BLAST. According to alignment results, 16S rRNA region isolated from the 49th population had 99% similarity with *R. sphaeroides*. Also the evolution tree in Figure 3.5 shows the similarity of the query searched. Lastly, growth curve of the 64th population was obtained to monitor the growth characteristics of the last population of selection and compare it with those of the reference strain. Cultures were monitored in pure M27 and M27 containing 4 mM cobalt. The OD₅₃₅ values obtained periodically were plotted in Figure 3.7. The results indicated that there was no significant growth behaviour difference between the 64th population and the reference strain, when there is no cobalt in the medium. However, in medium containing 4 mM cobalt, a clear difference between the evolutionary engineered the 64th population and reference strain was observed. While the 64th population succeeded in maintaining almost the same growth characteristics as in the medium without cobalt, the reference strain showed a significant decrease in its growth rate. It took nearly 20 h for the mutant strain to reach the stationary phase of growth, while the reference strain required about 60h to reach the stationary phase. These differences showed the obvious alteration in the metabolism of 64th population, and made clear that the cobalt-resistant final population was not inhibited by 4 mM cobalt, which significantly reduces growth of the reference strain.

To conclude, this study verified that evolutionary engineering is a rapid and efficient method for improving cobalt tolerance of the metabolically diverse bacterium *R. sphaeroides*. This study is the first application of an evolutionary engineering strategy in *R. sphaeroides*. Selection of mutant individuals from the final (64th) population of selection, and their physiological and genetic characterization are necessary to understand the molecular basis of cobalt resistance in this microorganism. Based on previous results with *S. cerevisiae*, it is highly expected that the final mutant population may be a heterogeneous one (Cakar et al., 2012), which emphasizes the

need for careful analysis of mutant individuals to be obtained from this final population.

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