

CHARACTERIZATION OF THREE PARTIALLY CONSERVED AMINO ACID
MUTATIONS IN *CBB₃*-TYPE OXIDASE ENZYME OF *RHODOBACTER*
CAPSULATUS

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GÜLGEZ GÖKÇE YILDIZ

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Prof. Dr. Yaşar DÜRÜST
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Doctor of Philosophy.

Prof. Dr. Ekrem GÜREL
Head of Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy.

Assist. Prof. Dr.Mehmet ÖZTÜRK
Supervisor

Examining Committee Members

Prof. Dr. Sezai TÜRKEK

Assoc. Prof. Dr. İbrahim ÇAKIR

Assoc. Prof. Dr. Serap MUTUN

Assoc. Prof. Dr.Azra BOZCAARMUTLU

Assist.Prof. Dr. Mehmet ÖZTÜRK

ABSTRACT

CHARACTERIZATION OF THREE PARTIALLY CONSERVED AMINO ACID MUTATIONS IN *CBB₃*-TYPE OXIDASE ENZYME OF *RHODOBACTER CAPSULATUS*

Yıldız, Gülgez Gökçe

Ph.D., Department of Biology

Supervisor Assist. Prof. Dr. Mehmet ÖZTÜRK

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Heme-Copper Oxygen reductases are responsible for catalyzing the reduction of oxygen to water and generate an electrochemical gradient, which is used for ATP synthesis. *cbb₃*-type oxidases are the most distinct members of the HCOs which are characterized by high catalytic activity at low oxygen concentrations and reduced proton pumping. Analyses on genomic sequences for C-type oxygen reductase family and mutagenesis studies on conserved residues showed that each of these conserved residues has a critical role in structure and/or function of the enzyme. In this study, three partially conserved residues Asparagine 265, Tyrosine 420 and Phenylalanine 480, which are unique to C-type oxygen reductase family, were substituted to Valine, Phenylalanine and Alanine respectively by site directed mutagenesis for the investigation of the diversity, mechanism and structure of this

highly diverse family. Characterization of the mutants from *Rhodobacter capsulatus* was done with respect to their effects on activity, assembly and proton pumping stoichiometry. Mutations at the position of N265 and Y420 showed that, they have critical role on the enzyme activity and assembly while F480 has role in function, proton translocation. Findings support the assumption that partially conserved residues may also have important role in the structure and/or function of the *cbb*₃-type oxidases.

Keywords: *Rhodobacter capsulatus*, respiration, cytochrome *cbb*₃-type oxidase activity, site-directed mutagenesis, chromatophore membrane proteins.

ÖZET

***RHODOBACTER CAPSULATUS*'IN *CBB₃*-TİPİ SİTOKROM OKSİDAZ ENZİMİNİNDE KISMİ OLARAK KORUNMUŞ ÜÇ AMİNOASİT MUTASYONUNUN KARAKTERİZASYONU**

Yıldız, Gülgez Gökçe

Doktora, Biyoloji Bölümü

Tez Danışmanı: Yrd. Doç. Dr. Mehmet ÖZTÜRK

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Demir-Bakır Oksijen (DBO) redüktazlar oksijeni suya indirgemekten ve ATP sentezi için kullanılacak olan elektrokimyasal farkı yaratmaktan sorumludurlar. *cbb₃*-tipi oksidazlar, DBO ailesinin en farklı üyeleri olup, düşük oksijen konsantrasyonunda yüksek aktivite gösterme ve düşük proton pompalama özellikleri ile karakterize olurlar. Süper aile içerisinde yapılan sekans karşılaştırmaları ve korunmuş aminoasitler üzerine yapılan mutasyon çalışmaları *cbb₃*-tipi oksidazların, süper ailenin diğer üyeleri ile bazı ortak özelliklere sahip olduğunu göstermiştir ancak bu enzimin çalışma mekanizması halen çok iyi bilinmemektedir. Bu çalışmada, oldukça çeşitlilik gösteren C-tipi oksijen redüktaz ailesinin çalışma mekanizmasını, yapısını ve çeşitliliğini incelemek amacıyla, bu aileye özgü kısmi olarak korunmuş olan üç aminoasit, asparajin 265, tirozin 420 ve fenilalanin 480 yönlendirilmiş mutagenез yöntemi ile sırasıyla valin, fenilalanin ve alanin'e

dönüştürülmüştür. *Rhodobacter capsulatus*'a ait cbb_3 -tipi oksidaz mutantları enzim aktivitesi, yapısı ve proton pompalama sitokiyometri özelliği bakımından incelenerek karakterizasyonları yapılmıştır. N265 ve Y420 pozisyonundaki mutasyonların enzim aktivitesine ve yapısına etki ettiği, F480 mutasyonunun ise proton translokasyonu üzerinde etkisi olduğu bulunmuştur. Araştırma sonuçları kısmi olarak korunmuş olan aminoasitlerinde cbb_3 - tipi oksidaz enzimi üzerinde yapı ve/veya fonksiyonel olarak önemli yere sahip olduğunu göstermiştir.

Anahtar kelimeler: *Rhodobacter capsulatus*, solunum, cbb_3 tipi sitokrom c oksidaz aktivitesi, yönlendirilmiş mutagenез, kromatofor zar proteini

To my grandparents, Ayten-Arslan ARARAT

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ABBREVIATIONS

Å	Angstrom (10 ⁻¹⁰ meters)
Amp ^R	Ampicilline resistance
APS	Ammonium persulfate
ATP	Adenosine-5'-triphosphate
BCA	Bicinchoninic acid
BCIP/NBT	5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt/nitro-blue tetrazolium chloride
BN-PAGE	Blue native polyacrylamide gel electrophoresis
bp	Base pair
CcO	Cytochrome c oxidase
CO	Carbon monoxide
DDM	n-Dodecyl β-D-maltoside
DMPD	N', N'-dimethyl-p-phenylenediamine
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ETC	Electron Transfer Chain
FAD	Flavin adenine dinucleotide, oxidized form

FADH ₂	Flavin adenine dinucleotide
FMN	Flavin prosthetic group
HCO	Heme- copper oxygen reductases
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)
ICM	Intracytoplasmic membranes
IPTG	Isopropyl –β-D-thiogalactopyranoside
Kan ^R	Kanamycin resistance
kbp	Kilo base pair
KCN	Potassium cyanide
kDa	Kilo Dalton
LB	Luria-Bertani
MedA	Minimal medium
mg	Milligram
ml	Milliliter
MOPS	3-(N-morpholino) propanesulfonic acid
MW	Molecular weight
NADH	Nicotinamide adenine dinucleotide, reduced form
NADP	Nicotinamide adenine dinucleotide phosphate
Ni-NTA	Nickel nitrilotriacetic acid
ng	Nanogram
NO	Nitric oxide

NORs	Nitric Oxidase Reductases
PCR	Polymerase chain reaction
PMF	Proton motive force
PMSF	Phenylmethanosulphonylfluoride
PS	Photosynthetic
PVDF	Polyvinylidene fluoride
RT	Room temperature
SAP	Shrimp alkaline protease
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Spect ^R	Spectinomycin resistance
TB	Transformation Buffer
TBST	Tris-Buffered Saline and Tween 20
Tet ^R	Tetracycline resistance
TMBZ	3,3',5,5'-tetramethylbenzidine
TMPD	Tetramethylphenylenediamine
UQ	Ubiquinone
UQH ₂	Ubiquinol
WT	Wild type
X-Gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside
μ g	Microgram
μ l	Microliter

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CHAPTER 1

INTRODUCTION

1.1. Energy Transfer through Living Organisms

All living things, from the basic unit of life, the cell, to a higher order of cells require energy to perform energetically expensive life processes like growth and reproduction. On the other hand even non-growing cells need energy to maintain themselves. As the energy cannot be created or destroyed, cells have no way to produce energy; however they exist systems which have capabilities for extracting energy from respective interstitial, host, or ambient environments. In those systems, same energy can also be converted to the biologically useful forms. As the Sun is the ultimate source of all the energy that powers the life, plants and photosynthetic organisms utilize the energy of photons from the Sun and convert it to the chemical energy in organic molecules by the process of photosynthesis. The energy stored in the bonds of organic molecules is transformed to the more usable form by the process of cellular respiration which involves the breaking apart the bonds of organic molecules. The free energy derived from breaking apart the bonds of organic molecules and from light is partially converted to a carrier molecule called adenosine triphosphate (ATP) and used by cells. Altogether photosynthesis and cellular respiration form a closed cycle in which energy can circulate and transform among living things.

1.2. *Cellular Respiration and Electron Transfer Chain*

Cellular respiration, in which cells extract energy stored in organic molecules and transfer that energy to the molecules of ATP and used it for cellular activities. The chemical reactions of this process occur in three steps. In first step, glycolysis takes place in cytoplasm of the cell and involves the conversion of glucose into three-carbon molecules of pyruvate with generation of ATP and NADH. During glycolysis ATP is produced by substrate level phosphorylation which caused a small yield of ATP. The organisms use anaerobic metabolism do not follow the next steps of cellular respiration, they stop at this point. Other two stages of the cellular respiration take place in mitochondria in eukaryotes. As the prokaryotic organisms lack mitochondria, the reactions for aerobic respiration occur in the cytosol in an association with the cytoplasmic membrane. In the second stage, each pyruvate enters mitochondria and converted acetyl coenzyme A which is finally oxidized to carbon dioxide. In the course of Krebs cycle, one molecule of ATP is produced by substrate level phosphorylation like in glycolysis. Addition to ATP, one molecule of FADH_2 and three molecules of NADH are produced which are used by the enzymes of the respiratory chain as electron donors in the next step of cellular respiration. In the last stage of cellular respiration, while the electrons are passed from one electron acceptor to another, energy is released during this flow and ETC uses this energy to pump protons across the inner membrane of mitochondria in eukaryotes (or across the plasma membrane in prokaryotes), forming a proton gradient. ETC does not produce ATP directly, sets the conditions for ATP production by chemiosmosis. The mystery of the connection between ATP production and ETC was dissolved by the chemiosmosis theory of Peter Mitchell in 1961 (Mitchell, 1961). According to this

theory, chemiosmosis uses potential energy stored in the form of a proton (H^+) gradient to phosphorylate ADP and produces ATP.

The respiratory chain consists of four membrane-bound protein complexes (Figure 1.1) which is located in the internal membrane of the mitochondria and the plasma membrane of aerobic bacteria. As the energy of the redox drop between NADH oxidation and O_2 reduction is higher than the membrane resistance, the energy of the redox drop is divided and stored in enzyme complexes.

The first complex (NADH: ubiquinone oxidoreductase or NADH dehydrogenase) is the largest and most complicated enzyme of the respiratory chain, containing 45 different subunits (Carroll et al., 2006) with flavin prosthetic group (FMN) and seven iron-sulfur clusters (Fe/S-4Fe/4S and 2Fe/2S types) which serve as redox cofactors (Sazanov and Hinchliffe, 2006). Complex I is an entrance point of electrons which transfers two electrons from NADH to coenzyme Q (ubiquinone) by flavin mononucleotide prosthetic group and via a series of iron-sulfur clusters (Efremov et al., 2010). During this process, for each electron two protons are translocated from the mitochondrial matrix to the intermembrane which produces a proton gradient (Bogachev et al., 1996).

Ubiquinone/ubiquinol pool (UQ/UQH₂ or Coenzyme Q or Q) is a system in which e^- and H^+ are carried by hydrophobic mobile carriers. UQ reduced to UQH₂ by accepting electrons and protons from Complex I and II transfer them to Complex III.

Complex II (succinate: ubiquinone oxidoreductase or succinate dehydrogenase) is composed of four subunits with covalently bound FAD, three 2Fe/2S centers and

heme *b*. This complex delivers additional electrons to reduced quinone pool, which are originated from succinate and transferred via FAD. Even this complex serves as entrance of electrons it is not involved in proton translocation across the membrane.

The third complex (ubiquinol: cytochrome *c* oxidoreductase or cytochrome *bc*₁ complex) contains 11 subunits with redox centers two heme *b*, a membrane anchored cytochrome *c*₁ and a 2Fe/2S center (Iwata et al., 1998). This enzyme complex catalyzes the oxidation of reduced coenzyme Q. Two electrons are removed from ubiquinol and transferred to cytochrome *c* which coupled with the transformation of four protons into the intermembrane space, per two-cycle process, known as Q-cycle (Mitchell, 1976; Crofts, 2004).

Cytochrome *c*, a soluble protein, has function as transporting electrons from Complex III to Complex IV.

The terminal oxidase, complex IV (cytochrome *c* oxidase) catalyzes the final steps of electron transport by transferring two electrons from cytochrome *c* to molecular oxygen, producing two molecules of water. Mitochondrial CcO is composed of 13 subunits with four redox cofactors; two hemes (*a* and *a*₃), bimetallic copper site-Cu_B and another copper center-Cu_A. Proton translocation across the membrane also occurs as a result of this reaction, in which four protons are removed from the mitochondrial matrix and two of them are translocated across the membrane, contributing to the proton gradient.

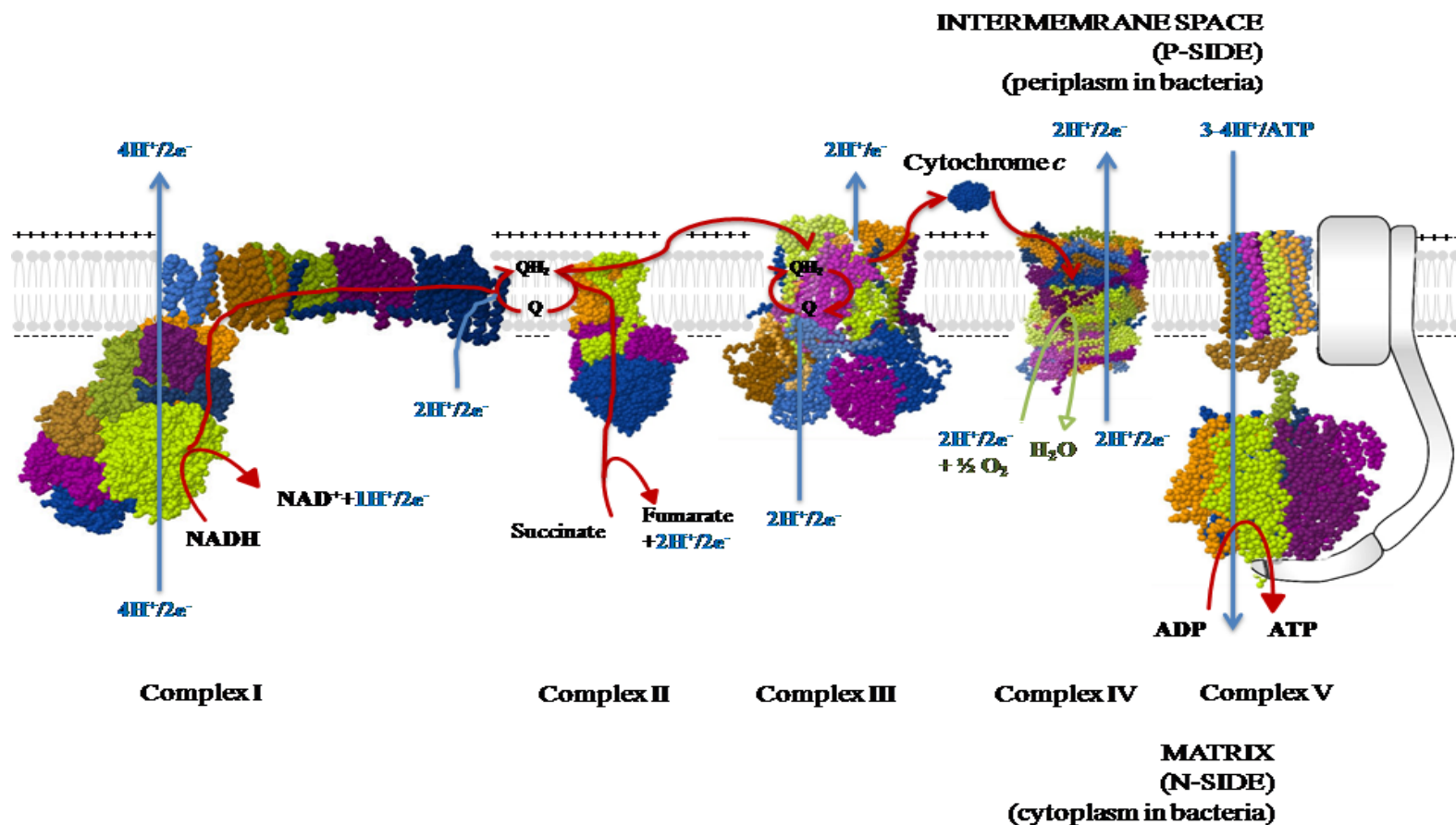


Figure 1.1 The respiratory chain of the mitochondria with ATP synthase. The structures represented are taken from protein database, (PDB) entries; PDB ID: 3M9S (complex I) (Efremov et al., 2010), PDB ID: 1Y3Q (complex II) (Huang et al., 2006), PDB ID: 1BGY (complex III) (Iwata et al., 1998), PDB ID: 2B4Z (cytochrome c) (Mirkin et al., 2008), PDB ID: 2DYY (complex IV) (Shinzawa-Itoh et al., 2007), PDB ID: 1QO1 (complex V) (Stock et al., 1999) figures was made by using Jmol software (<http://www.jmol.org/>).

1.3. Terminal Oxidases

1.3.1. General Function

Cytochrome *c* oxidase, as the terminal enzyme of the respiratory chain, catalyzes the reduction of oxygen to water. The overall reaction catalyzed by the enzyme can be summarized as:



$$n = \frac{\text{H}^+_{\text{PUMP}}}{\text{e}^-}$$

For the reduction of oxygen to water, four electrons, supplied one-by-one from cytochrome *c*, react with the enzyme at the outer surface of the membrane, P-side (for eukaryotic enzyme, mitochondrial inter-membrane space and for prokaryotic enzyme, bacterial periplasmic side) and eight protons per O₂ are taken up from the inside, N-side (for eukaryotic enzyme mitochondrial matrix and for prokaryotic enzyme bacterial cytoplasm), of which four of them are used in the chemistry of oxygen reduction. As the protons and the electrons are coming from different sides of the membrane to make H₂O from O₂, a net transfer of four charges across the membrane is taken place. This mechanism is explained as charge separation by vectorial chemistry, in which for each electron transferred to the active site, one charge crosses the membrane. In addition to vectorial chemistry mechanism, enzyme uses free energy, released in the reaction of oxygen reduction, for proton pumping

across the membrane. In proton pumping, four of the eight protons are taken from the N-side, transported across the membrane and released on the P-side (Wikstrom, 1977).

By vectorial chemistry and proton pumping properties of cytochrome *c* oxidase enzyme, transmembrane electrochemical proton gradient is generated and maintained across the membrane which is an efficient source of energy to make ATP, solute active transport and mechanical movement.

1.3.2. Classification

All examined aerobic bacterial species have branched respiratory systems including multiple terminal oxidases (Anraku, 1988), contrary to eukaryotic mitochondrial systems which have single terminal oxidase (Garcia-Horsman et al., 1994a). Containing more than one terminal oxidase provides advantages for bacterial species to live in different environmental conditions by regulating the intracellular oxygen concentration, rapid removal of excess reducing equivalents and maintaining the maximum coupling efficiency (H^+/e^-) (Garcia-Horsman et al., 1994a). The terminal oxidases are divided into three families according to their spectroscopic and structural observations and also for their ability to pump protons (Figure 1.2) (Borisov et al., 2011).

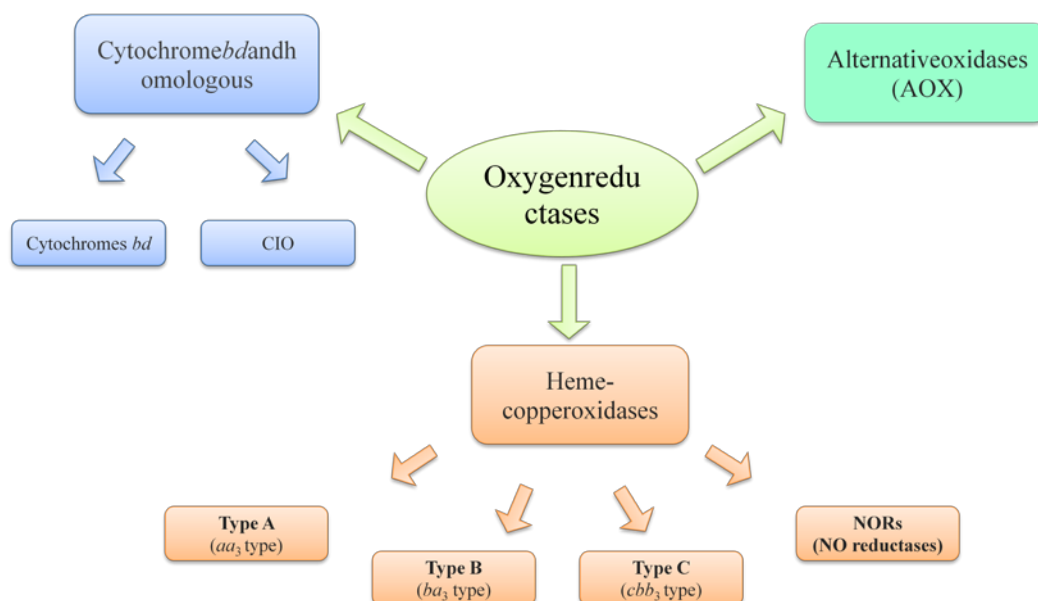


Figure 1.2 Diversity of terminal oxidases

The first, most extensively studied and largest the family is the Heme-Copper Oxidases which have a low spin heme and a binuclear center for the O_2 reduction composed of a high-spin heme (a_3 , o_3 or b_3) and a copper ion Cu_B with histidine ligand covalently linked to a tyrosine residue (Hemp et al., 2008). Members of this family generates proton motive force (PMF) by transmembrane charge separation to pump protons across the membrane (Wikström and Verkhovsky, 2007). The second family of the oxygen reductases is the cytochromes *bd* type quinol oxidases. These oxidases are generally found in bacteria (Anraku, 1988) and contain three hemes in their structure and two of them high spin hemes (heme b_{595} and heme d) are constitute the catalytic center for O_2 reduction (Hill et al., 1993; Arutyunyan et al., 2008; Borisov et al., 2011). Similar to heme copper oxidase family, cytochrome *bd* type oxidases generates PMF; however they do not pump protons (Miller and Gennis, 1985; Belevich et al., 2005). The third family of oxygen reductases comprises cyanide- resistant alternative oxidases (AOX) found in mitochondria of

higher plants, fungi and protists including pathogenic organisms (Albury et al., 2009). Alternative oxidases use ubiquinol as an electron donor for the reduction of oxygen to water and contain a non-heme di-iron carboxylate active site. In contrast to other oxidases, AOX do not generate PMF and also they do not have role in transmembrane charge transfer (Borisov et al., 2011). AOX have role in pollination and production of heat and the volatilisation of insect attractants (Wagner et al., 2008) and also there are other proposed roles for AOX which include the maintenance of TCA cycle and protection against oxidative stress (Albury et al., 2009).

1.4. *Heme-Copper Oxidases*

1.4.1. Evolution of Heme-Copper Oxidases

The Heme-Copper Oxidase (HCO) superfamily is a large and diverse superfamily which plays important roles in both aerobic and anaerobic respiration (Garcia-Horsman et al., 1994a; Pereira et al., 2001). Due to their essential roles on the aerobic respiration, understanding the origin and evolution of the members of HCOs is important to comprehend evolutionary mechanism of aerobic respiration on earth. In 1994 and 1995, Castresana and colleagues analyzed phylogenetic and taxonomic relationships of the major class of oxygen reductases; Heme-Copper Oxidases by using limited number of sequences (Castresana et al. 1994; Castresana & Saraste 1995; Castresana et al. 1995). According to these studies, three distinct subfamilies of HCOs were identified and it has been shown that phylogenetic trees do not follow the phylogeny of the organisms that based on the comparison of the 16S rRNA, which implies several gene duplications or lateral transfer events or both

happened during the evolution of the protein. The first proposal by Castresana and his/her colleagues based on the assumption that NO was present in the atmosphere prior to the O₂ and suggested that Nitric Oxidase Reductases (NORs) were first enzymes to appear and HCOs were derived from these enzymes (Saraste and Castresana, 1994). It was also proposed that, HCOs were found in a common ancestor of Bacteria and Archea, which was called as 'uroxidase', and was present before the appearance of the first photosynthetic eubacteria and thus before the increase of oxygen in the atmosphere due to oxygenic photosynthesis. By the first gene duplication FixN oxidases (C-type oxidases, *cbb₃*-type oxidases) were evolved from the rest of the enzymes which are functional under low oxygen conditions and the second gene duplication resulted in the evolution of other two oxidases (Castresana et al., 1994).

By growing the large number of complete genome sequences in recent years accurate phylogenetic studies have been performed to clarify the evolutionary history of HCOs. Pereira and colleagues (2001) classified HCOs into three major types and agreed the conception of Castresana and his/her colleagues (Castresana et al., 1994; Castresana and Saraste, 1995) about HCOs existing before the development of the oxygenic photosynthesis, later on this proposal was accepted by other studies, such as (Gribaldo et al., 2009; Sousa et al., 2012). In contrast to previous hypothesis about the origin of HCOs, it was proposed that HCOs did not have a common ancestor such as uroxidase, and they originated in Gram positive bacteria and were later transferred to Archea by horizontal gene transfer (Pereira et al., 2001). In 2012, Sousa and colleagues suggested that using only molecular phylogenies is not suitable without a proper root for the determination of the origin and the evolution of

the HCOs, a common denominator in HCOs would allow a better understanding of the evolution of these enzymes(Sousa et al., 2012).

1.4.2. Classification of Heme-Copper Oxidases

Saraste (1990) claimed that most respiratory oxidases from species ranging from microbes to humans are members of a single superfamily, called the Heme-Copper Oxidase superfamily. Members of the superfamily are present in eukarya, archaea and eubacteria and they are divided into two classes based on the chemical reaction that they form; (i) oxygen reductases and (ii) nitric oxide reductases. While oxygen reductases catalyze the reduction of molecular oxygen to water, nitric oxide reductases (NORs) catalyze the reduction of nitric oxide (NO) to nitrous oxide (N₂O) during denitrification (Zumft, 2005). It has been also shown that NORs have role in nitric oxide detoxification in some pathogenic bacteria (Philippot, 2005). In contrary to oxygen reductases, NOR are not able to pump protons and do not generate an electrochemical gradient across the membrane(Flock et al. 2006; Reimann et al. 2007). However, they share similar structural properties with oxygen reductases.

The HCOs can be divided into different subgroups with respect to their electron donors; (i) cytochrome *c* oxidases, which use *c*-type cytochrome by metal center, Cu_A, in the subunit II and include the mitochondrial respiratory oxidases and many bacterial oxidases and (ii) quinol oxidases, which use quinol directly and include many bacterial oxidases.

The superfamily members are quite diverse with respect to their subunit composition, heme types, electron donor and O₂ affinity (Table 1) (Garcia-Horsman et al., 1994a; Pereira et al., 2001; Pitcher and Watmough, 2004; Ducluzeau et al.,

2008). Membership and classification of the superfamily are based on the common features of the conserved core subunit (subunit I) and key residues in the proton conducting channels.

The conserved core subunit (subunit I) is composed of 12 transmembrane helices and harbors a binuclear metal center, composed of a high spin heme (a_3, o_3 or b_3) and a copper ion (Cu_B) with histidine ligand of Cu_B covalently linked to a tyrosine residue and a low spin heme (a or b type) (Garcia-Horsman et al., 1994a; Pereira et al., 2001; Hemp et al., 2006). In bacteria, several types of hemes, like B, O or A, can be found as redox cofactors (Calhoun et al., 1994; Garcia-Horsman et al., 1994a) (Figure 1.3). Heme O and Heme B have very similar absorption properties both reflects red color, heme O differs from heme B by containing the hydroxyethylfarnesyl side chain. Heme A contains a formyl group instead of methyl group of both Heme B and O, which resulted in the green absorption in the spectrum (Garcia-Horsman et al., 1994a) structure of heme C is similar to heme B except that heme C is covalently bound to polypeptide via thioether bridges.

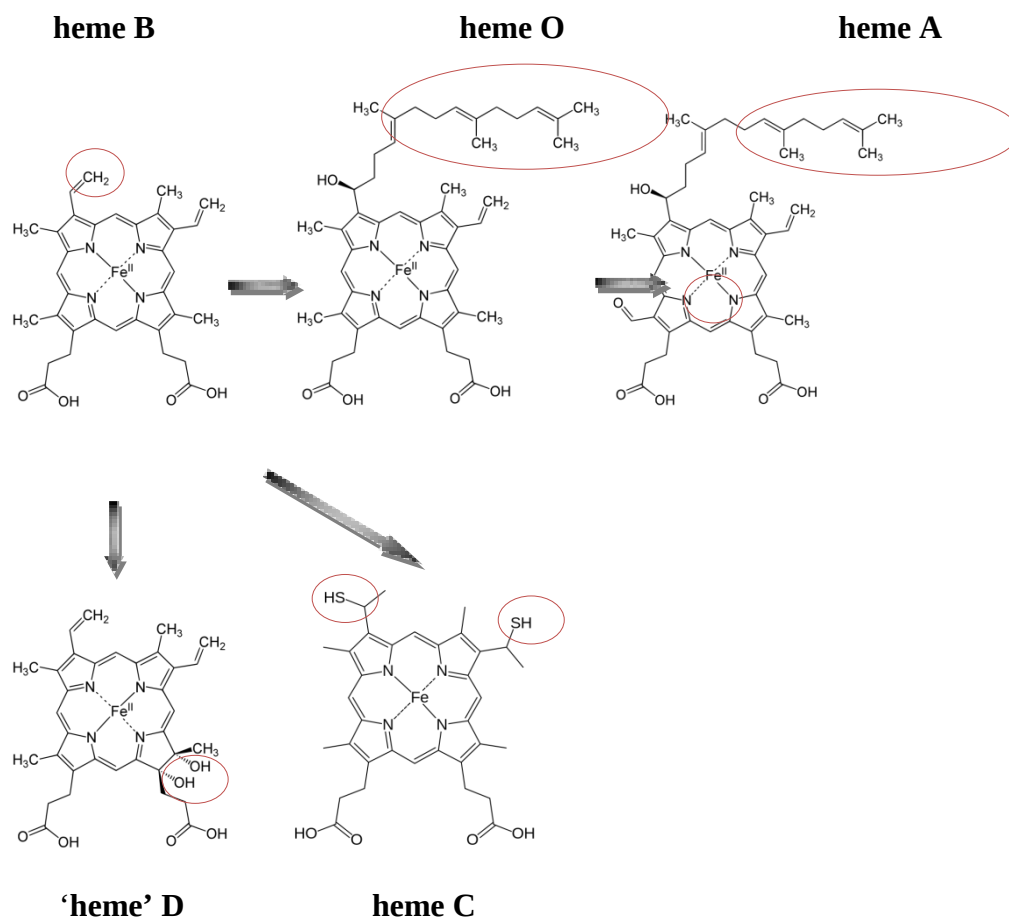


Figure 1.3 Structures of hemes. Structures of heme B, heme O, heme A, heme D (is a chlorine rather than a real heme), and heme C.

According to these common features HCO superfamily has been classified into three major subfamilies; A-, B- and C-type (Pereira et al., 2001) and also five additional subfamilies (D-,E-,F-,G-,H-) were proposed by Hemp and Gennis(2008). However, recent studies with large number of sequences revealed the existence of additional subfamilies and they are included in B-type family (Sousa et al., 2011, 2012). The A-type oxidases are further divided into two types, A1 and A2 types depending on the presence or absence of conserved glutamate residue in one of the proton channels (D- channel) (Pereira et al., 2001). Recent phylogenetic studies were also support this classification of HCOs (Sousa et al., 2011) (Figure 1.4).

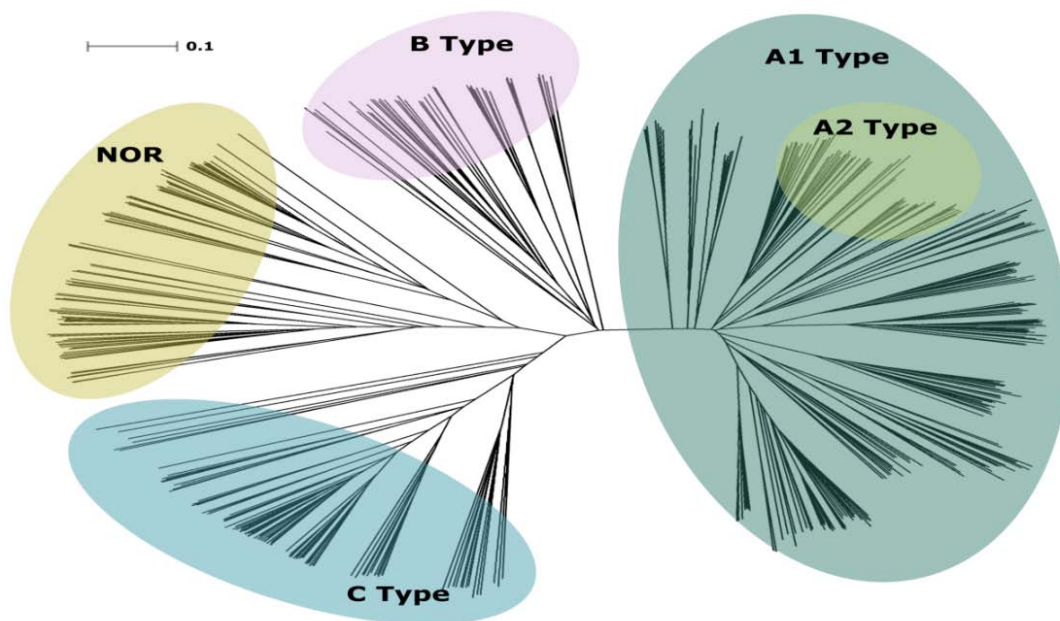


Figure 1.4 Phylogenetic analysis of HCOs. Phylogenetic analysis (neighbor-joining tree representation) of subunit I of HCOs and NOR enzymes (Sousa et al., 2011).

The largest and the best known HCOs are of the type found in mitochondria, the A-type family, which is widespread in bacteria, archaea and eukarya (Pereira et al., 2001; Gribaldo et al., 2009; Sousa et al., 2012). The first structural information for A-type family came from the best studied bovine mitochondria and *Paracoccus denitrificans* aa_3 (Tsukihara et al., 1996; Iwata et al., 1998), followed by *Escherichia coli* bo_3 (Abramson et al., 2000), and *Rhodobacter sphaeroides* aa_3 (Svensson-Ek et al., 2002; Qin et al., 2006). The functional core subunit of this family is composed of two subunits, subunit I and subunit II. The redox cofactor Cu_A , bound to the subunit II, accepts the electrons from soluble cytochrome *c* and transfers them to the active site, composed of a high spin heme a_3 and Cu_B ion, via low spin heme *a* in subunit I. The members of the A-type family are characterized by two proton conducting channels, D- and K- channels (Pereira et al., 2001), which are used for the transport of chemical and pumped protons. The A1-type D- channel starts at the cytoplasmic surface with, Asp-124 (D) (*P. denitrificans* numbering) and continues with

hydrophilic amino acid residues (Asn-199, Asn-113, Asn-131, Tyr-35, Ser-134, Ser-193) and ending with key residue Glu-278. The residues, Lys-354 (K), Thr-351, Ser-291 and Tyr 280 are constitute the K-channel of A1-type family (Pereira et al., 2001) A2-type includes all the residues compose both D and K- channels with the exception of Glu-278 in D-channel. This key residue is replaced with Tyr and Ser next to each other in sequence, and this YS motif is the fingerprint of A2-types (Pereira et al. 1999; Pereira et al. 2000). H- channel in bovine *aa₃* (Lee et al., 2000) and E-channel in *P. denitrificans aa₃* (Pfitzner et al., 1998) have been proposed as additional channels however no experimental data support the presence of these channels and also these channels were not observed in the other members of the family (Sousa et al., 2012).

Second family named as B-type is the least represented family among the HCOs however they are present in both archaea and bacteria (Sousa et al., 2012). The most studied and structurally defined member of the B-type family is *ba₃* oxidase from *Thermus thermophilus* (Soulimane et al., 2000). Subunit I of the B-type family is composed of 13 transmembrane helices and 12 of which are homologues to the subunit I of A-type. Second subunit, subunit II, contains Cu_A site similar to A-type family. In B-type oxidases, the residues constituting the D- and K- channel of A-type are missing. The pattern of conserved residues revealed that a K- channel analogue is present (Chang et al., 2009) with a threonine, a serine and a tyrosine residues replacing Lys-354, Thr-351, Ser-291, respectively. Two other alternative channels, D and Q, were proposed for these enzymes, however sequence alignments showed that the residues which compose these proposed channels are not common among the B-

types (Pereira et al., 2001). The B-type family is also characterized by their higher affinity for O₂ in the comparison with A-type oxidases (Han et al., 2011).

The C-family oxygen reductases, known as the *cbb*₃- type oxidases, is the second largest oxygen reductase family after A-family (Hemp and Gennis, 2008) and members of this family are found mainly in bacteria (Gribaldo et al., 2009; Sousa et al., 2012). Members of this superfamily have been purified and characterized from *P. denitrificans*(De Gier et al., 1996), *Bradyrhizobium japonicum*(Preisig et al., 1996b), *Rhodobacter sphaeroides*(Garcia-Horsman et al., 1994b; Oh and Kaplan, 1999), *Vibrio cholerae*(Hemp et al., 2005) and *Pseudomonas stutzeri*(Pitcher et al., 2002) and recently the structural information is obtained from *P. stutzeri*(Buschmann et al., 2010). The catalytic subunit of C-type family is CcoN which is similar to the subunit I of A- and B-types. These reductases apparently have only part of the alternative K-channel observed for the B-type enzymes, with a serine and a tyrosine in the place of the Thr-351 and Ser-291(Pereira et al. 2001; Hemp et al. 2007; Sousa et al. 2012). Like B- type oxidases, C-type members have high affinity to oxygen (Preisig et al., 1996b). Unlike the A- and B- type families, similar to NORs, members of the C-family are able to reduce NO to N₂O(Forte et al., 2001).

General characteristics of the HCOs family members are summarized in Table 1.1 and available 3D structures are shown in Figure 1.5.

Table 1.1 Characteristics of members of Heme–Copper Oxygen reductase families

	A-type family (<i>aa</i> ₃ -type)	B-type family (<i>ba</i> ₃ -type)	C-type family (<i>cbb</i> ₃ -type)	NOR
Subunit composition	• Subunit I* • Subunit II with Cu_A • Subunit III • Subunit IV • Other subunits	• Subunit I * • Subunit II with or without CuA	• CcoN* • Two <i>c</i>-type cytochromes : CcoP and CcoO • Other small subunit: CcoQ	• NorB* • <i>c</i>-type cytochrome: NorC
Catalytic binuclear center	• heme <i>a</i>₃ • heme <i>a</i> • Cu_B • Covalent His-Tyr	• heme <i>a</i>₃ • Cu_B • Covalent His-Tyr	• heme <i>b</i>₃ • heme <i>b</i> • Cu_B • Covalent His-Tyr • Ca²⁺ coordinating heme <i>b</i> and heme <i>b</i>₃ propionates	• heme <i>b</i>₃ • heme <i>b</i> • Non-heme iron Fe_B • No covalent His-Tyr • Ca²⁺ coordinating heme <i>b</i> and heme <i>b</i>₃ propionates
Enzymatic properties	K _m 0.7-2 μM	K _m ~0.1 μM	K _m ~7 nM (for O ₂) K _m ~12 mM (for NO)	
Proton pathway	K pathway D Pathway (in A1 E) (in A2 YS)	K pathway	K pathway	Protons from periplasm

* Catalytic subunit of the enzyme

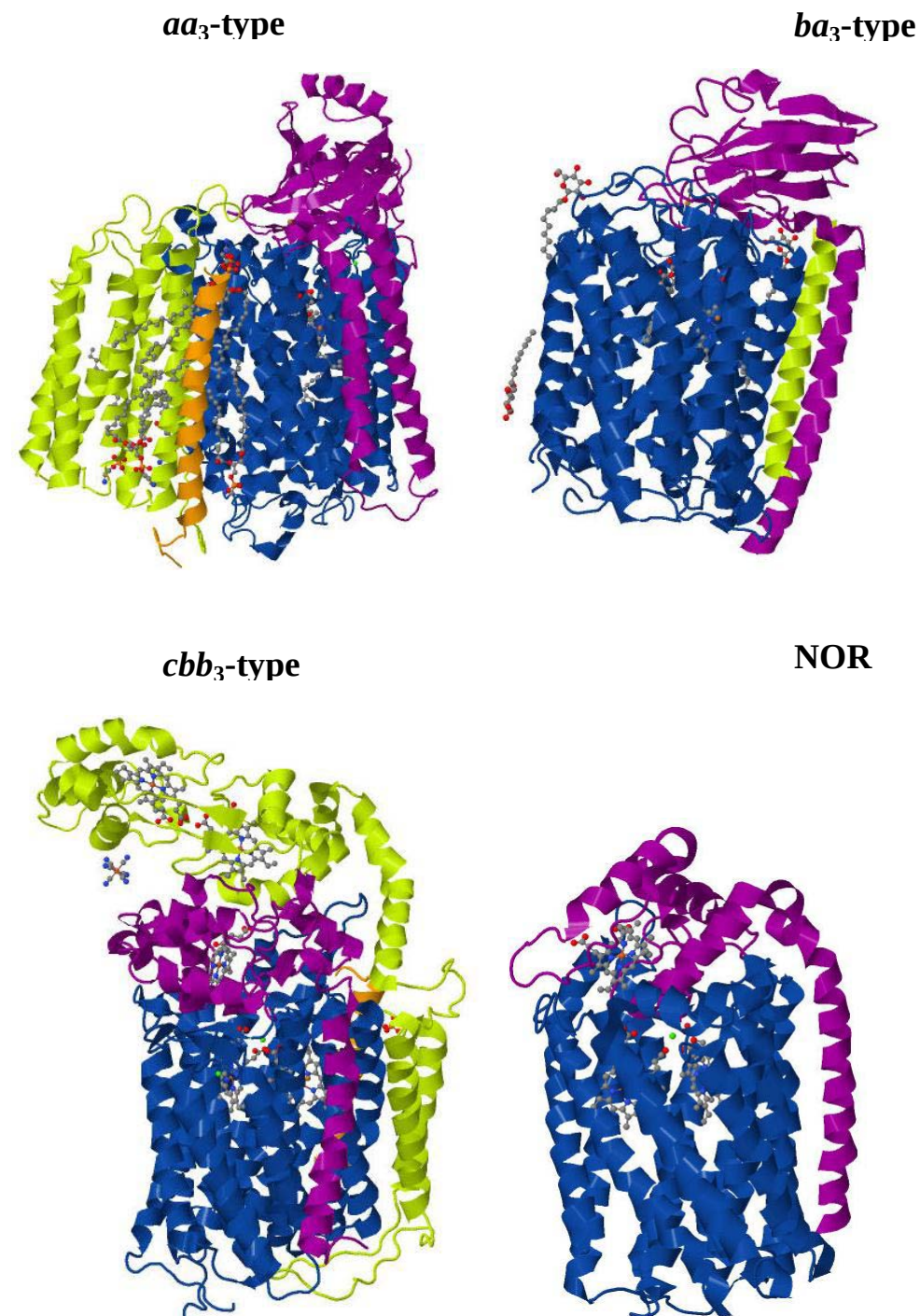


Figure 1.5 3D structures of Heme-Copper Oxygen Reductases. Catalytic subunit I blue (*cbb*₃-type CcoN), subunit II (*cbb*₃-type CcoO); magenta, subunit III (*cbb*₃-type CcoP); green, additional subunit; orange. Spheres: copper; blue, heme iron; dark gray, non-heme iron; light gray, calcium; yellow. The structures represented are taken from protein database, (PDB) entries; PDB ID: 1M56 (*aa*₃-type) (Svensson-Ek et al., 2002) PDB ID: 1EHK (*ba*₃-type) (Soulimane et al., 2000), PDB ID: 3MK7 (*cbb*₃-type) (Buschmann et al., 2010), PDB ID: 3O0R (NOR) (Hino et al., 2010) and figures were made by using Jmol software (<http://www.jmol.org/>).

1.5. The *cbb*₃-type Oxygen Reductases

1.5.1. Distribution of *cbb*₃-type Oxygen Reductases in Bacteria

According to the sequence similarities of the catalytic subunit, CcoN, the *cbb*₃-type enzymes are found mainly in Proteobacteria. Proteobacteria are phylogenetically divided into α , β , γ , δ and ϵ subgroups. The $\alpha\beta\gamma$ proteobacterial subgroups include *cbb*₃ oxidases from *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, *Pseudomonas stutzeri*, *Bradyrhizobium japonicum*, *Rubrivivax gelatinosus*, *Vibrio cholera*, *Neisseria gonorrhoeae* and *Neisseria meningitides*. *cbb*₃ oxidases from *Helicobacter pylori* and *Campylobacter jejuni* belong to ϵ subgroup (Ducluzeau et al., 2008). In addition to Proteobacteria, *cbb*₃ oxidases are found in Aquificae, Acidobacteria, Bacteroidetes, Chlorobi, Firmicutes and Spirochaetes phyla. (Figure 1.6). As the *cbb*₃ oxidases have high affinity for O₂, many human pathogenic bacteria such as *Campylobacter jejuni* (Parkhill et al., 2000), *Helicobacter pylori* (Tomb et al., 1997), *Neisseria gonorrhoeae* and *Neisseria meningitides* (Li et al., 2010) have only *cbb*₃-type oxidase in their respiratory chain as a terminal oxidase which allows these pathogens to colonize at low O₂ containing tissues (Pitcher and Watmough, 2004).

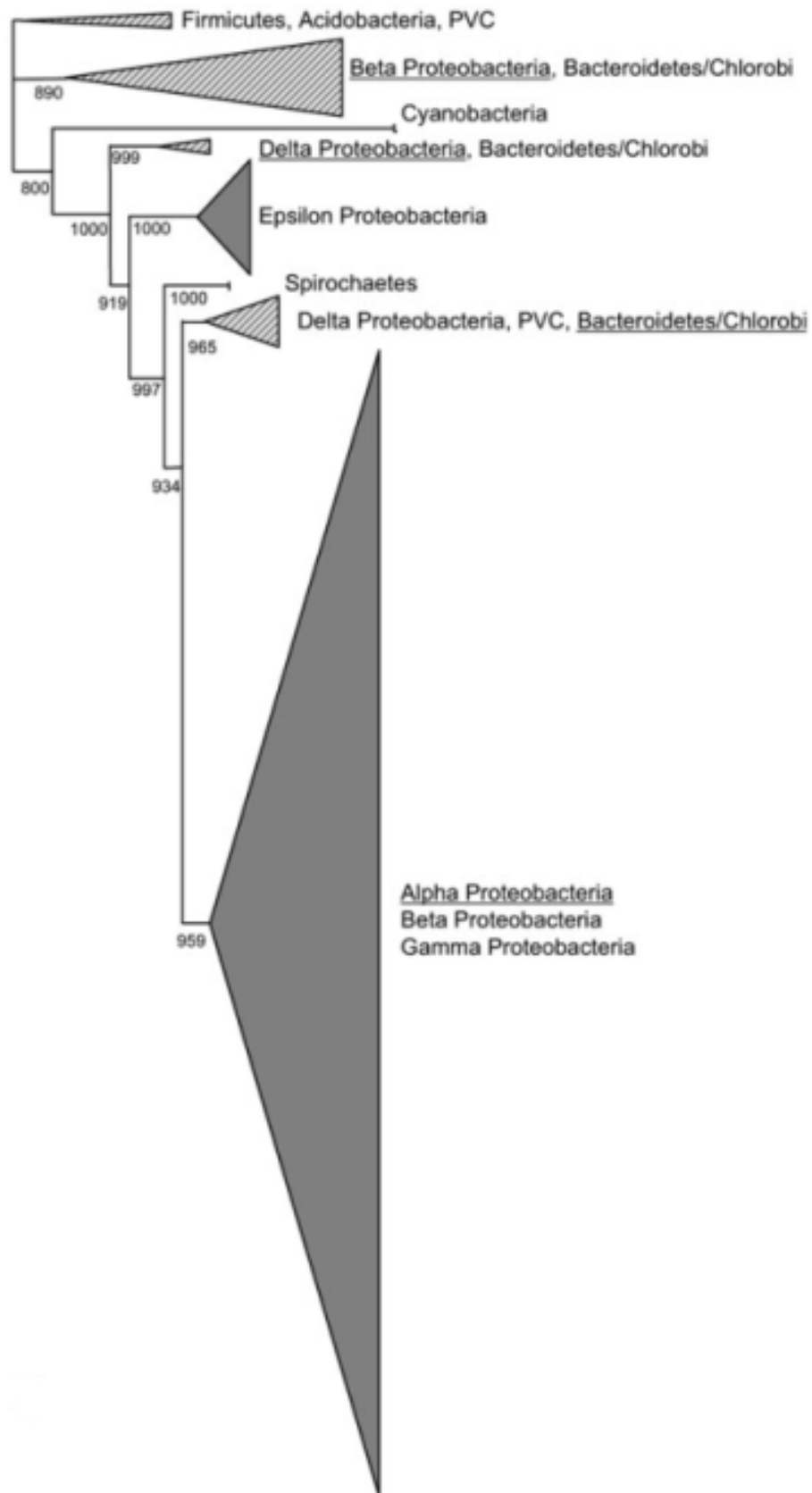


Figure 1.6 Schematic representation of the unrooted Maximum Likelihood (ML) tree of the catalytic subunit of *cbb*₃-type oxygen reductases (Brochier-Armanet et al., 2009).

1.5.2. *Rhodobacter capsulatus* and its Respiratory Chain

The *Rhodobacteraceae*, family of Proteobacteria, mainly called as purple non-sulfur bacteria, includes an interesting organism, *Rhodobacter capsulatus* due to its versatile metabolic pathways. Gram negative *R. capsulatus* cells are rod-shaped (0.6-0.8 x 1.3-1.6 μm) nonmotile, divide by binary fusion or budding and form zigzag chains which has not been observed in any other *Rhodobacter* species (Brenner et al., 2005). As mentioned above, *R. capsulatus* has a number of metabolic pathways to grow under different environmental conditions by performing aerobic respiration, anaerobic respiration, photosynthesis and fermentation (Imhoff, 1995) (Figure 1.7).

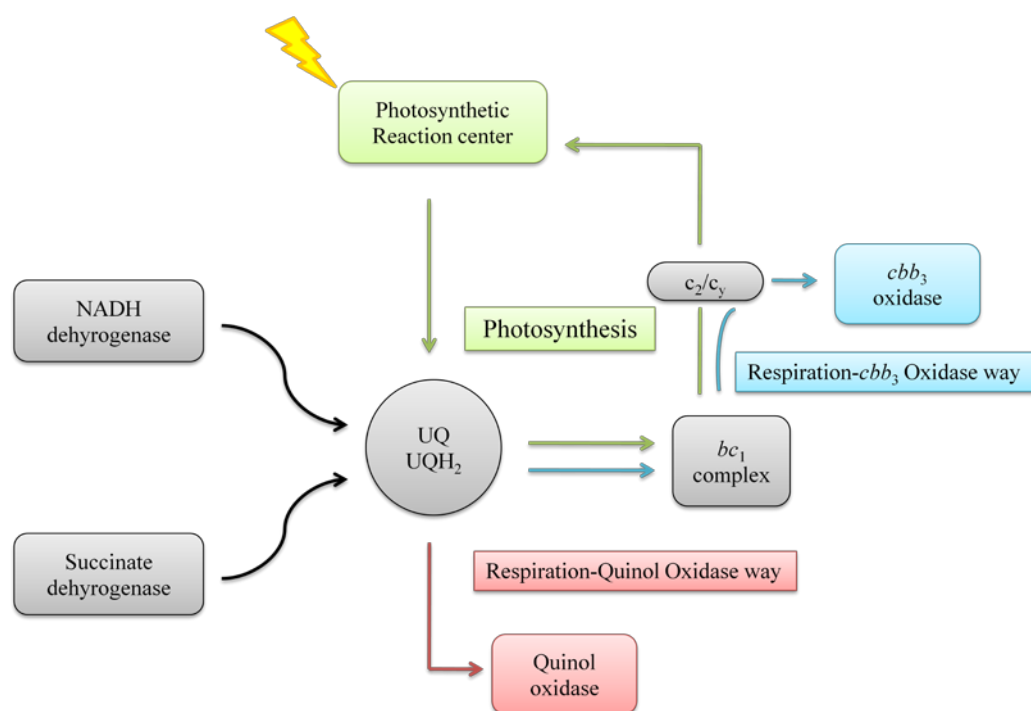


Figure 1.7 The respiratory chain of *R. capsulatus*. UQ and UQH₂ correspond to the oxidized and reduced form of quinones. Soluble cytochrome c₂ and the membrane-bound cytochrome c_y are electron donors for the photosynthetic reaction center and cbb₃ oxidase. In photosynthetic pathway, shown by green arrows, electrons come from reaction center which is activated with photon and transferred to Q pool and then to cytochrome bc₁ complex and electron cytochrome c₂ or cytochrome c_y and return back to the reaction center again. In respiratory pathway, electrons come from NADH and Succinate dehydrogenases and transferred to Q pool. After this step, electrons may follow cbb₃ Oxidase way, shown by blue arrows, which includes cytochrome bc₁ complex, cytochrome c₂ or cytochrome c_y and cytochrome cbb₃ oxidase or Quinol Oxidase way, shown by red arrows, which includes quinol oxidase (Modified from (Ekici et al., 2012)).

Under photosynthetic (PS) conditions bacteria can grow photoautotrophically with H_2 or low levels of sulfide as electron donors; in PS pathway electrons circulate in a cyclic process between the photochemical reaction center and the bc_1 complex (Meyer and Donohue, 1995) by using Q pool and electron carriers cyt c_2 (Daldal et al., 1986) and cyt c_y (Jenney and Daldal, 1993).

R. capsulatus can grow well under chemoautotrophic conditions aerobically in the dark with molecular hydrogen as electron donor, O_2 as electron acceptor and CO_2 as carbon source (Madigan and Gest, 1979). In some cases, like under oxic dark conditions it can also use sulfide as electron donor (Kompantseva, 1981). It can be also grown with vigorous aeration for oxygenation (Madigan, 1988). In respiratory pathway, electrons are transferred from respiratory dehydrogenases, NADH, Succinate and Malate dehydrogenase, to the Q pool, and after the quinone pool, the respiratory electron transfer pathways of *R. capsulatus* are branched into two different terminal oxidases that convert O_2 to H_2O (Koch et al., 1998). The cbb_3 oxidase branch is composed of similar electron carriers and protein complexes, cytochrome bc_1 complex, cytochrome c_2 or cytochrome c_y , of the PS pathway except final electron acceptor, cytochrome cbb_3 oxidase which includes two c -type cyts, c_o and c_p , as subunits (Gray et al. 1994; Koch et al. 1998). In this pathway, electrons shuttle from the cytochrome bc_1 complex to cytochrome cbb_3 oxidase via cytochrome c_2 , as well as cytochrome c_y (Daldal, 1988; Hochkoeppler et al., 1995). In the quinol oxidase pathway, electrons pass to the quinol oxidase directly without using any electron carriers.

As *R. capsulatus* known as the most metabolically versatile species among of all known bacteria (Madigan and Gest, 1979), it has been chosen as an excellent

model for studying microbial energy transduction (Zannoni, 1995). The referred variable metabolic pathways of *R. capsulatus* provide an advantage for detecting the functional *c*-type cytochromes phenotypically, by the ability of cells catalyzing the α -Naphthol plus *N*', *N*'-dimethyl-*p*-phenylenediamine (DMPD) plus O₂ → indophenol blue plus H₂O reaction (NADI reaction). If any of the cytochromes, *c*₁, *c*₂ and *c*_y, is absent, PS pathway will be blocked on the other hand in the absence of *c*_o and *c*_p cytochromes, *cbb*₃ pathway will be blocked and both of these situations can be tested by NADI reaction (Marrs and Gest, 1973) and *R. capsulatus* can survive by using other pathways.

1.5.3. *ccoNOQP* Operon and Regulation of *cbb*₃-type Oxidase Expression

The structural genes encoding the *cbb*₃-type oxidase complex were isolated from rhizobial species, *Bradyrhizobium japonicum*, and named as *fixNOQP* for their role in symbiotic nitrogen fixation (Batut et al., 1989; Preisig et al., 1993). Subsequent studies identified these genes and their products also in non-symbiotic, gram negative bacteria such as *R. capsulatus*, and referred them *ccoNOQP* (Thöny-Meyer et al., 1994; Gray et al., 1994; Myllykallio and Liebl, 2000). The regulation of terminal oxidase expression in *R. capsulatus* is dependent on the environmental O₂ conditions. It has also been shown that the activity of the *cbb*₃ oxidase is related with O₂ levels, in which activity is high under microaerobic, low under fully aerobic, and even lower under fully anaerobic growth conditions (Swem et al., 2001). The regulatory system that is composed of response regulator RegA sensor and kinase RegB respond to different O₂ conditions and regulate the photosynthesis gene expression in *R. capsulatus* (Sganga and Bauer, 1992; Mosley et al., 1994). Under

microaerobic and aerobic conditions the dephosphorylated form of RegA activates *cbb*₃-type oxidase expression. Conversely, under anaerobic conditions RegA is phosphorylated by the sensor kinase RegB, and phosphorylated RegA represses *cbb*₃-type oxidase and enhances photosynthesis genes expression (Swem et al., 2001). In addition to RegA and RegB, *fnr*-like genes, *fnrP* and *fnrL*, found upstream of *ccoNOQP* in *R. capsulatus* (Koch et al., 1998) also regulate the *cbb*₃-type oxidase expression. It has been demonstrated that inactivation of FnrL in *R. capsulatus* decreases *cbb*₃-type oxidase activity by about 80% under anaerobic growth conditions, suggesting that it acts as an activator in the absence of O₂ and this effect is counter balanced by the trans-acting regulatory protein HvrA, which functions as a repressor of *ccoNOQP* under microaerobic and anaerobic conditions (Swem and Bauer, 2002). Another regulatory system for *cbb*₃-type oxidase expression is FixLJ-K regulatory system in which, FixL can bind O₂ via a heme *b* group and cause the phosphorylation of FixJ (a transcription factor) under low oxygenic conditions the phosphorylated FixL activates the expression of FixK (a downstream transcription regulator) and FixK binds specifically upstream of *ccoN* gene to activate its expression (Rey and Harwood, 2010; Ekici et al., 2012).

1.5.4. Subunit Composition and Structure of *cbb*₃-type Oxidase

The *cbb*₃-type oxidase enzymes are composed of CcoN (subunit I), CcoO (subunit II), CcoQ (subunit IV) and CcoP (subunit III) proteins (Ducluzeau et al., 2008) which are encoded by the *ccoNOQP* operon. Buschmann and colleagues (2010) solved the 3D structure of *cbb*₃-type oxidase from *P. stutzeri* with 3.2 Å resolution. The overall structure revealed that CcoN (the catalytic subunit) is localized in the

membrane like other members of HCOs; however heme containing subunits, CcoO and CcoP, are found mainly in periplasm in contrast to other oxidases (Figure 1.8).

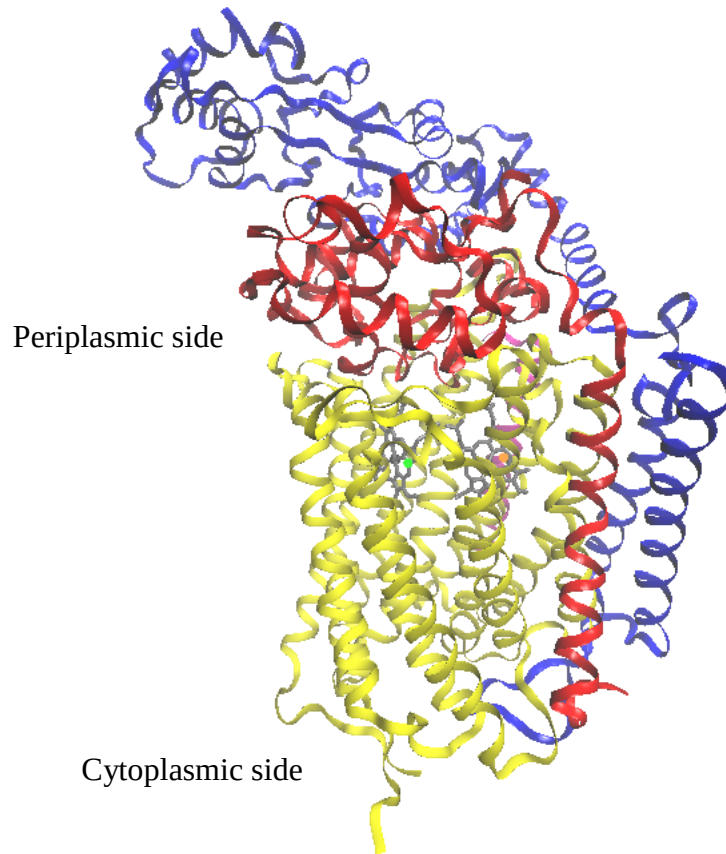


Figure 1.8 General structure of *cbb*₃-type oxidase. General structure of *cbb*₃-type oxidase from *Pseudomonas stutzeri*. (PDB ID: 3MK7 (Buschmann et al., 2010)) with CcoN in yellow, CcoO in red, CcoQ in magenta and CcoP in blue. Heme groups are in silver, iron ions in black, copper ions in orange, calcium ions in green and target residues, N265, Y420 and F480 are in red, cyan and purple respectively. This figure was generated by using VMD software, version 1.9.1 (Humphrey et al., 1996).

1.5.4.1. Subunit I (CcoN)

CcoN subunit is composed of 12 transmembrane (α 1- α 12) helices with its amino (N-) and carboxyl (C-) termini located close to each other on the cytoplasmic side (Gray et al., 1994; Buschmann et al., 2010). The predicted molecular mass is 45 kDa (Garcia-Horsman et al., 1994b). The catalytic center is located near the outer

membrane surface and consists of a low spin heme *b*, a high spin heme *b*₃ and a Cu (Cu_B) atom. According to the structural data, heme *b*₃ has a more bent conformation compared to the other HCOs and the iron of heme *b*₃ is ligated to His-345 (*P. stutzeri* numbering) at its proximal side and an unknown ligand from its distal side. In contrast to other HCOs, this His-345 residue is hydrogen bonded to Glu-323 and then hydrogen bonded to Trp-341 and Trp-400. Heme *b*₃ and heme *b* are connected together via a calcium ion by coordinating their carboxyl groups of pyrole D rings. Similar to the other HCOs, three histidines surround the Cu_B atom and one of them, His-207, is covalently link to the Tyr 251. This catalytically important tyrosine residue is found on helix VII not on helix VIII like in other HCOs. Cu_B atom is also ligated to the unknown ligand of heme *b*₃. Two calcium atoms are found in the structure, one of these atoms interacts directly with the heme *b*₃-Cu_B catalytic center, while the second one is located between the loops IV and V at the edge of the structure (Figure 1.9).

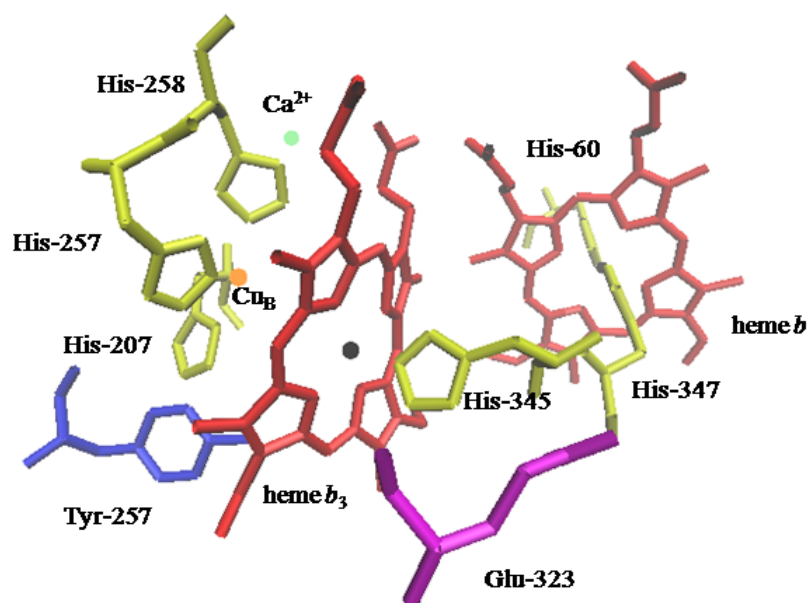


Figure 1.9 The catalytic binuclear center of *cbb₃*-type oxidase. The catalytic binuclear center of *cbb₃*-type oxidase from *Pseudomonas stutzeri*. (PDB ID: 3MK7 (Buschmann et al., 2010)). Heme-red, His-yellow, Tyr-blue, Glu-magenta. Spheres: copper -orange, heme iron-black, calcium-green. This figure was generated by using VMD software, version 1.9.1 (Humphrey et al., 1996).

1.5.4.2. Subunit II (CcoO)

CcoO with the molecular mass of 28 kDa (Toledo-Cuevas et al., 1998) is composed of one N-terminally located transmembrane helix and a soluble cytochrome *c* domain carrying heme *C* molecule. CcoO connects the first two conserved helices, binds to heme *b₃*, heme *b* and heme *c*, and especially contacts the calcium atom that coordinates heme *b* and heme *b₃* at the catalytic center via its Ser102 residue. Also, it interacts with the loops helix III-IV, helix V-VI, helix VII-VIII, helix IX-X and helix XI-XII of CcoN. Addition to its interactions with CcoN, it has interaction with several residues of the subunit P (Buschmann et al., 2010). The role of CcoO is to convey electrons to heme *b* of CcoN.

1.5.4.3. Subunit III (CcoP)

In the structural data of *P.stutzeri* it has been showed that CcoP consists of two N-terminal transmembrane helices with long linker that makes multiple contacts to the cytoplasmic part of CcoN and a soluble globular unit built up by two cytochrome domains with one heme *c* bound to each. On the other hand, for *R. capsulatus* it is proposed that CcoP has only one transmembrane helix which act as an anchor (Ekici et al., 2012). The 32 kDa subunit CcoP (Garcia-Horsman et al., 1994b) has role in accepting electrons by its distal heme group from a soluble or membrane bound electron donor (in *R. capsulatus*, cytochrome *c*₂ or cytochrome *c*_y) and transfers them to the proximal heme of this subunit and then to CcoO which also transfers to the CcoN. It has also been showed that the proton entrance is located in CcoP (Lee et al., 2011). Together with CcoO, CcoP has an important role in the assembly of active *cbb*₃-type oxidase(Peters et al., 2008).

1.5.4.4. Subunit IV (CcoQ)

The CcoQ with molecular weight of 7.7 kDa (Toledo-Cuevas et al., 1998), is a small subunit formed by a single transmembrane helix. It has not got any cofactor. In the structural data CcoQ is absent and shown as a helix located close to helices IX and XI of CcoN. Even several studies has been performed in *B. japonicum* (Zufferey et al., 1996) and *R. sphaeroides*(Oh and Kaplan, 1999) about the functional and structural importance of this subunit, no apparent effect upon the assembly or the activity of *cbb*₃-type oxidase has been detected thus the overall function of this subunit is unclear.

1.5.5. Assembly of *cbb*₃-type Oxidases

The assembly of cytochrome c oxidases is defined as complex process because of containing multiple cofactors and the location of the enzyme in the membrane (Cobine et al., 2006). While the assembly of *aa*₃-type in eukaryotes requires more than 30 cofactors, bacterial *aa*₃-type oxidases require less. The assembly of *cbb*₃-type oxidases is mediated by a set of genes, located immediately downstream of *ccoNOQP* operon, *ccoGHIS* (Koch et al., 1998).

ccoH, in *ccoGHIS* gene cluster, encodes a singlespanning membrane protein with an extended periplasmic domain that is suggested to serve as a dimerization domain. According to the BN-PAGE analysis of active *cbb*₃-type oxidase detectable in *R. capsulatus* membrane, the molecular weight was calculated as 230 kDa (Kulajta et al., 2006). Assembly of the 230 kDa complexis composed of two inactive assembly intermediates which are a 210 kDa and a 40 kDa subcomplexes. First subcomplex contains the catalytic subunit CcoN, the mono-heme cytochrome *c* subunit CcoO and CcoH (Kulajta et al., 2006; Pawlik et al., 2010). The second subcomplex is composed of the di-heme cytochrome *c* subunit CcoP, the small subunit CcoQ and a second copy of CcoH (Pawlik et al., 2010). Full assembly of CcoNOH and CcoQPH subcomplexes is achieved by the dimerization of the cytoplasmic domains of the two CcoH(Kulajta et al., 2006).

The other genes, *ccoS*, *ccoI* and *ccoG*, in the *ccoGHIS* gene cluster have role in the incorporation of hemes*b* and Cu into CcoN. CcoS, a small membrane protein, has role maturation of CcoN by incorporating heme *b* into CcoN. In the absence of CcoS, an inactive *cbb*₃-type oxidase assembles that lacks both of the heme *b* cofactors of

CcoN but contains properly matured CcoO and CcoP subunits (Koch et al., 2000). The essential role of CcoI for *cbb*₃-type oxidase assembly suggests a model that involves transport of Cu⁺ from the cytoplasm to the periplasm, then its concomitant or subsequent insertion into CcoN (Ekici et al., 2012). On the other hand, the exact function of CcoG is unknown however it has been proposed that it might be involved in intracellular oxidation of Cu⁺ to Cu²⁺ (Preisig et al., 1996a) or CcoG might reduce Cu²⁺ to Cu⁺ to provide the substrate for CcoI (Ekici et al., 2012).

1.6. Pathways in HCOs

The main function of Heme-Copper Oxidases is the reduction of molecular oxygen to water which occurs in the binuclear center of the enzyme. As this binuclear center is buried in the middle of the enzyme, it requires specific pathways for the delivery of electrons, protons and oxygen as substrates and release of the product, water. These pathways can be classified as electron-, proton- and oxygen transfer pathways and water exit channels.

1.6.1. Electron Transfer Pathways

Four electrons are required for the reduction of molecular oxygen to water and these electrons are supplied one by one from water soluble cytochrome *c* which serve as one electron mediator between *bc*₁ complex and cytochrome *c* oxidase. Cytochrome *c* binds to the enzyme between subunits II and III on the P-side of the membrane (Fuller et al. 1981; Iwata et al. 1995). By electrostatic interactions between cytochrome *c* and cytochrome *c* oxidase, and hydrophobic forces (Pelletier and Kraut, 1992) the *c*-CcO complex is formed. In A- and B- type oxidases after cytochrome *c* binds to the enzyme the electron is instantly transferred to the primary

electron acceptor, Cu_A (Kobayashi et al., 1989). According to the electron transfer theory the distance between electron and acceptor is an important criteria for electron transfer rate and efficiency (Marcus and Sutin, 1985). In the case of electron transfer from Cu_A, even heme *a(b)* and heme *a*₃ share the similar distance from Cu_A, the preference is shifted to heme *a*, which might be explained by thermodynamics. Afterwards the electron is transferred from heme *a(b)* to heme *a*₃ without coupled proton translocation. The final step of the electron transfer includes the transfer of electron to Cu_B from heme *a*₃ with coupled proton transfer. After the second electron is transferred to binuclear center, O₂ binds to the heme *a*₃ iron, reduced and cleaved by receiving electrons from the heme *a* iron, Cu_B and redox active cross-linked tyrosine residue.

The electron transfer of *cbb*₃-type oxidase completely differs from A- and B-type oxidases, which might also affects electron transfer rates, catalysis and proton pumping (Buschmann et al., 2010). According to the crystal structure, electron is transferred to outer heme *c* molecule of subunit P from periplasmic cytochrome *c*. Then, electron is transferred to inner heme *c* molecule of the same subunit and from there to the heme *c* molecule of subunit O. Afterwards, electron is shuttled to heme *b* of subunit N from heme *c*. Electron transfer continues from heme *b* to heme *b*₃, and from heme *b*₃ to Cu_B and electron rapidly equilibrates with Cu_B. The binding of the O₂, reduction and cleavage are similar to other oxidases. However, structural data revealed that distance between heme *c* of subunit O and heme *b* is shorter than between Cu_A and heme *a(b)* in A(B)-type oxidases caused an increased transfer rate between them and faster trapping and reduction of O₂ (Verkhovsky et al., 1996). In addition, the conformation of the heme rings and propionates, the position of the

crosslinked tyrosine phenol group, and the environment of the histidine ligated to the heme b_3 iron might contribute to the lower K_M value for O_2 of cbb_3 -type oxidases (Buschmann et al., 2010).

1.6.2. Proton Transfer Pathways

In all cytochrome *c* oxidases catalytic site is embedded in the enzyme, thus protons required for the active-site chemistry and also for proton pumping require specific pathway(s). These channels connect the active site with the N-side of the membrane by containing hydrophilic amino acid residues with internal water molecules. Even the X-ray structures provide better understanding of the proton input pathways of the channels the output of the channels remain unclear (Brändén et al., 2006). The achievement of proton translocation by the enzyme has been proposed by using Grotthuss type mechanism, in which proton is transferred to an acceptor water molecule by means of O-H covalent bond breakage in the donor molecule and interacting with either backbone amide groups or hydrophilic side chains (De Grotthuss, 1806; Agmon, 1995; Cukierman, 2006).

The site-directed mutagenesis studies (Hosler et al., 1993; Fetter et al., 1995) and structural data analysis revealed (Tsukihara et al. 1996; Iwata et al. 1998) two proton pathways in A-type family, on the other hand studies on B- and C- type families showed that these families have only one proton input pathway (Hemp and Gennis, 2008; Chang et al., 2009; Buschmann et al., 2010; Soulimane et al., 2000). Available crystal structures of oxidases revealed that helices of subunit I are arranged symmetrically to produce three pores named as A (from helix III to VI), B (from helix VII to X) and C (helix XI-XII, I-II) (Tsukihara et al., 1996) (Figure 1.10).

responsible for the translocation of all four pumped protons and two chemical protons in the catalytic cycle (Konstantinov et al. 1997; Adelfroth et al. 1997).

The K-pathway, named after the conserved residue Lys-354, K354 (Hosler et al. 1993; Thomas et al. 1993) is found in pore B, formed by the helices VII-X (Lee et al., 2012). The K-pathway starts from Glu-78 in subunit II (Ma et al., 1999; Brändén et al., 2002) and continues with conserved residue such as, Thr-351 and Ser-291 to the Tyr -280 which is cross-linked to the one of the histine ligands of Cu_B. According to the crystal structures, two to three water molecules have been resolved within the pathway (Belevich, 2007). The K-pathway has role in uptake of another two chemical protons during the reductive part of the cycle. (Konstantinov et al., 1997; Adelfroth et al., 1998; Wikström, 2000).

A third pathway, H-pathway, has been suggested based on the structural and mutagenesis studies for mitochondrial A-type oxidases (Tsukihara et al., 1996; Yoshikawa et al., 2011). Even some residues are conserved in bacterial A-type oxidases, H-pathway is not functionally important in proton translocation (Lee et al., 2000).

The conserved residues of the single proton pathway of B- and C- type oxidases is located in pore B which is analogous to the K-pathway in the A family (Hemp et al., 2007; Chang et al., 2009; Buschmann et al., 2010). In B-type oxidases, the K-pathway most likely starts at the Glu-15 in subunit II, *T. thermophilus*ba₃ numbering) and continues through polar amino acids such as T312 and T315 up to the catalytic site (Lee et al., 2012). Site-directed mutagenesis studies (Chang et al., 2009) revealed that mutations in K-pathway were resulted in activity loss and

inhibition of the reduction of the active site heme, this results indicate that B-type family uses only one proton pathway.

In C-type family, K- pathway analogue is also found and started at Glu-49 (*P. stutzeri* numbering) which is located in CcoP and continues Ser-240, Tyr-223, His-243, Tyr-317, and Thr-215 to the cross-linked Tyr-251 that is $\sim 4 \text{ \AA}$ away from the O₂ binding site (Kaila et al., 2009; Buschmann et al., 2010). The mutagenesis studies (Yıldız et al., unpublished data) and structural modeling (Hemp et al., 2007) revealed that these conserved residues are important in proton pumping of C-type oxidases. The K-pathway in C-type family is slightly enlarged because of the different origin of the histidine-cross-linked tyrosine and the absence of the hydroxyethylfarnesyl substituent, which alters proton access to the O₂ binding site (Buschmann et al., 2010).

The structural data of cNORs revealed that there is no proton pathway which is analogue to K-pathway of A-, B- and C-type oxidases (Hino et al., 2010). As protons are required as substrate for the reduction of NO and the enzyme is also embedded into the membrane similar to the other HCOs, proton conducting pathway is required therefore a possible proton transfer pathway is proposed by Hendriks and colleagues (2002). This pathway started at the equivalent of the Glu-135 and continues with Asp-198, Ser-277, Glu-211 and Glu-280.

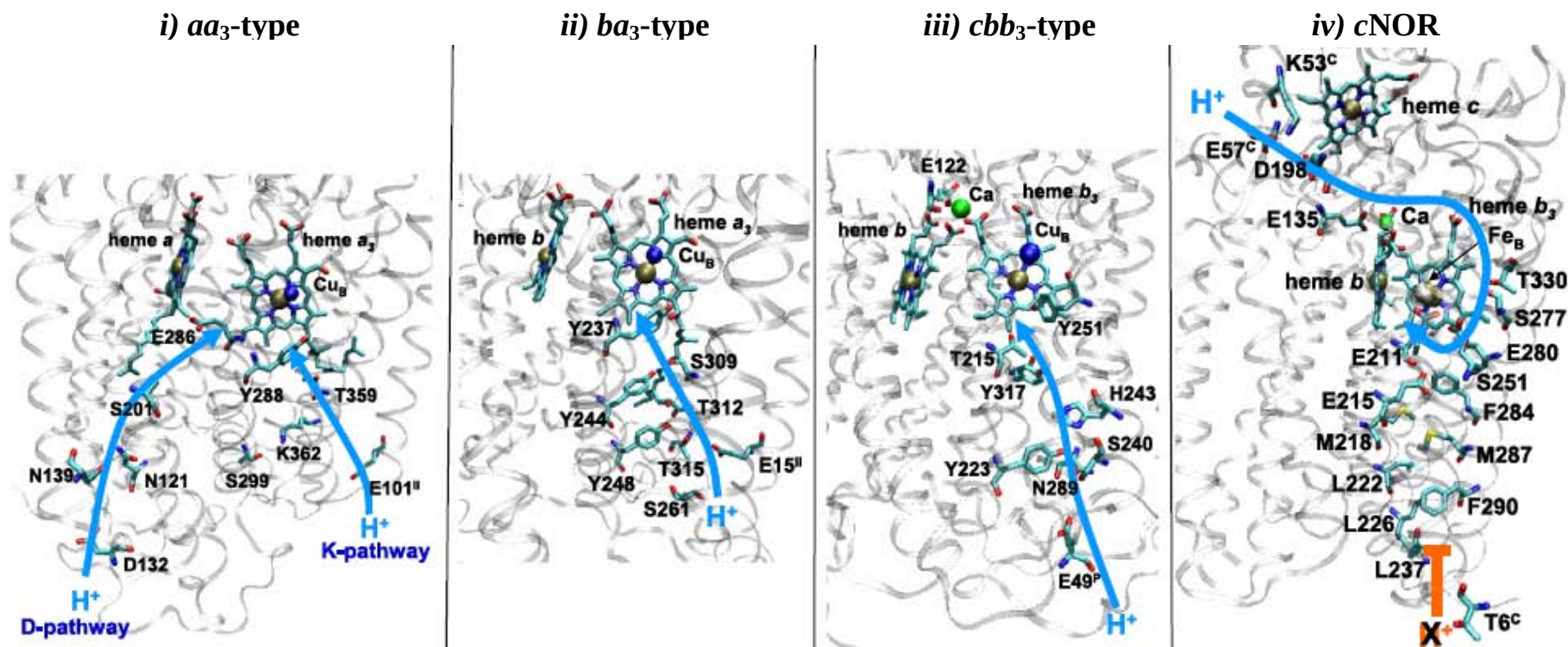


Figure 1.11 The proton pathways in the HCOs. **i)** the D- and K-pathways in the A-type oxidase from *R. sphaeroides* (Svensson-Ek et al. 2002). **ii)** The K-pathway analogue in the B-type oxidase from *T. thermophilus* (Soulimane et al., 2000). **iii)** The K-pathway analogue in the C-type oxidase from *P. stutzeri* (Buschmann et al., 2010). **iv)** One of the suggested proton pathways from the periplasm to the active site in cNOR from *P. aeruginosa* (Hino et al., 2010). Figure was taken from (Lee et al., 2012).

1.6.3. Oxygen Transfer Pathways

To maintain normal catalytic activity of the oxidases and the diffusion of O₂ into the protein, oxidases have certain structures that function as channels for oxygen delivery to the reaction center. For A- and B- type oxidases, structural (Tsukihara et al., 1996; Svensson-Ek et al., 2002) and experimental (Riistama et al., 1996, 2000; Salomonsson et al., 2004) computational data revealed that hydrophobic channels were found which are started in subunit II or III from the middle of the membrane bilayer, where oxygen is concentrated and flow through subunit I and reached to binuclear center via conserved Valine residue (Riistama et al., 2000). In the 3D structure of *cbb*₃-type oxidases two cavities were observed, one of them is embedded in the membrane and connected by narrow and hydrophobic channels to the binuclear active site, which possibly providing an O₂ access pathway (Buschmann et al., 2010).

1.6.4. Water Exit Channel

During each catalytic process of oxidases, two water molecules are produced in the binuclear center which must be removed from the enzyme. Florens and co-workers (2001) proposed a channel which is located immediately above the active site, at the interface of subunits I and II. In all eukaryotic CcO the region above the hemes contains a non-redox-active metal site containing aMg²⁺, this highly conserved Mg²⁺ ion lies at the bottom of the proposed water channel and bridges subunits I and II. According to the studies of Schmidt and colleagues (2003) water released from the active site reaches the Mn²⁺, it then follows the proposed channel (Florens et al., 2001) that contains residues along the interface of subunit I and

released at the P-side of the membrane. Thus water molecules has specific route that passes next to the $\text{Mg}^{2+}/\text{Mn}^{2+}$ binding site. In *cbb*₃-type oxidases, according to the structural data, the cavity which is close to the periplasmic face of the membrane, between CcoO and CcoN and located at a position equivalent to the end of the D-channel of type A enzymes, is proposed to provide an exit path for protons and water molecules from the catalytic site to the periplasm (Buschmann et al., 2010).

1.7. Reduction of O₂- Catalytic Cycle

The mechanism of O₂ reduction is extensively studied in A-type oxidases by using flow- flash technique in which fully reduced enzyme with carbon monoxide (CO) bound to the high-spin heme is mixed in a stopped-flow apparatus with an oxygenated solution. After that short laser flash is then applied, photolysing the Fe\CO bond and the binding of dioxygen and its subsequent step-wise reduction can be followed using time-resolved spectroscopy (Lee et al., 2012). Addition to spectrophotometric techniques, chemical studies have provided a clear picture of the mechanism of O₂ reduction to two water molecules with intermediates of the catalytic cycle. Catalytic cycle with intermediates is summarized in Figure 1.12.

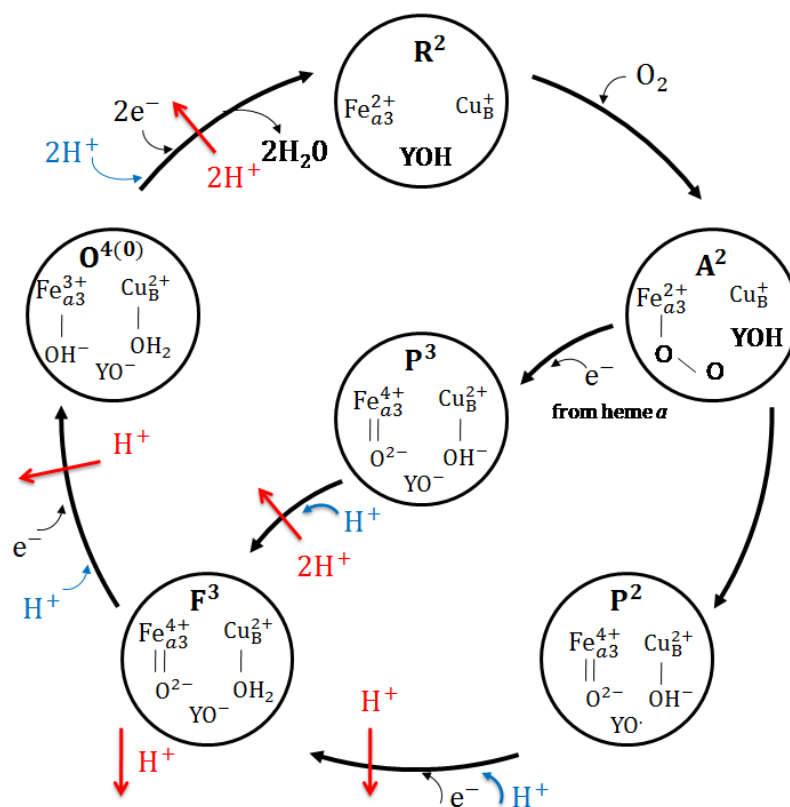


Figure 1.12 The catalytic cycle in A (1)-type HCuOs. The outer circle represents the reaction sequence during turnover, $R^2 \rightarrow A^2 \rightarrow P^3 \rightarrow F^3 \rightarrow O^{4(0)}$, where the superscript indicates the number of electrons transferred to the binuclear site. The YO_H is the Tyr cross-linked to one of the His ligands to Cu_B, which acts as the proton donor to the active site in the P³ state, and also as an electron donor in P². Proton uptake from bulk is indicated by blue arrows, the red arrows indicate which transitions are coupled to proton pumping across the membrane. Drawing is modified from (Lee et al., 2012)

Reduced (R) intermediate: The reaction with O₂ requires that the catalytic site be reduced by electrons. Heme a₃ and Cu_B, as the redox centers of the binuclear center, are reduced by two electrons- intermediate called as mixed valence-**MV**. When all four redox centers including Cu_A and heme a are reduced by electrons, then the intermediate is called as fully reduced **FR**. Both **MV** and **FR** are the starting point of the intermediates in the reaction cycle.

Ferrous-oxy (A) intermediate: After the enzyme reduced, O₂ first binds Cu_B and then to the heme a₃ (Woodruff et al. 1991; Lemon et al. 1993; Verkhovsky et al. 1994) forming the A intermediate with the time constant ~10 μs.

Peroxy (P) intermediate: In peroxy intermediate, O-O bond is broken which requires four electrons and a proton. The ‘peroxy’ intermediate can be present in one of two forms depending on the initial reduction state of the enzyme. If the enzyme is fully reduced (**FR**) for the O₂ reaction, P³ intermediate forms with three of the four electrons required from the catalytic site (two from heme a₃ which forms a ferryl and one from Cu^B) and the fourth from heme a (Lee et al., 2012). Time constant for the formation of P³ intermediate is ~30 μs (Verkhovsky et al. 1994; Adelroth et al. 1998). When the O₂ reaction is initiated with two electron reduced state (MV) P² intermediate is formed with a time constant ~150 μs (Hill & Greenwood 1984; Oliveberg et al. 1989; Varotsis et al. 1990) which contains a radical at a Tyrosine (Tyr 280, *P. denitrificans* numbering) crosslinked to the histidine ligand to Cu_B, as it donates the fourth electron (Proshlyakov et al., 2000). The proton requirement to break O-O bond is provided by the same tyrosine residue which is in its deprotonated form (YO⁻) in P³ and in a neutral radical form (YO[•]) in P².

Ferryl (F) intermediate: Taking the chemical proton from N-side of the membrane (via D- channel in A-types) to the catalytic center is caused the formation of Ferryl intermediate. Ferryl intermediate is formed from P² intermediate in with a time constant ~50 μs (Ribacka et al., 2005) and from P³ intermediate ~90 μs (Verkhovsky et al. 2006). In addition, one proton is pumped during the P→F (both P²→F and P³→F) transition (Brändén et al., 2006).

Oxidized (O) intermediate: O intermediate is the last intermediate in catalytic cycle which appears with a time constant $\sim 1\text{-}2\ \mu\text{s}$ (Hill and Greenwood, 1984; Oliveberg et al., 1989) from F intermediate and requires one electron and proton (via D- channel in A-types (Konstantinov et al., 1997)) to the catalytic center. The electron arrives at heme a_3 , and a ‘chemical’ proton arrives at the oxygenous ligand of heme a_3 , forming a hydroxyl, meanwhile a proton is pumped during the FO transition. During $\text{O} \rightarrow \text{R}$ transition, two electrons are required. During electron transfer reactions first Cu_B is reduced and then both Cu_B and heme a_3 are reduced. Each of these reactions is coupled to the uptake of two protons and release of two protons and the enzyme is returned to the R intermediate (Wikström and Verkhovsky, 2002; Bloch et al., 2004).

The overall O_2 reduction in A- type oxidases is described above however in B- and C- type oxidases there are some differences. In best studies B-type oxidase, cytochrome ba_3 from *T. thermophilus*, it has been shown that O_2 binding is 10 times higher than the A- type oxidases (Szundi et al., 2010) and the F intermediate is not observed in pH 7.5 which might be linked to the differences in proton pumping mechanism (Siletsky et al., 2007). In C-type oxidases, presences of three c-type hemes and complex CO binding behavior prevent getting clear identification of intermediates by flow flash technique (Lee et al., 2012). However, it has been shown that A intermediate is formed in C-type oxidases similar to the A-type oxidases and the formation of P^3 is also occurred (Lee et al., 2011). Also in C-type oxidases the CcoN lacks the equivalent of the tyrosine residue in the primary sequence, but another tyrosine, Tyr 257 (*P.stutzeri* numbering), on a different helix fulfills the same role (Hemp et al., 2005, 2006).

1.8. Aim of the Study

C-type oxygen reductases, *cbb*₃-type oxidases, are the most distinct members of the HCOs which are characterized by high catalytic activity at low oxygen concentrations and reduced proton pumping (Arslan et al., 2000; Han et al., 2011). This superfamily is quite different from other HCOs in terms of electron donors, subunit composition and also heme types (Pereira et al., 2001). Genomic sequences of prokaryotes for C-type oxygen reductase family were analyzed by Hemp et al., (2007) to identify conserved amino acid residues which may play important functional roles. According to this study there were completely conserved (99%) and partially conserved (95%) residues in C-Family of HCOs and they were classified into four categories. The first group includes the completely conserved five histidine residues in all members of the superfamily; second group was composed of the conserved residues within all members of the superfamily except NO reductases; residues that were conserved in C-type family and at least one of the other families of the superfamily were constitute the third group; and finally fourth group includes the conserved residues which were unique to C-type oxygen reductase family. Mutagenesis studies have been widely used for elucidating the importance of individual amino acid residues in protein structure, assembly and function. To date, highly conserved residues from C-type oxygen reductase family have been chosen as the sites of mutations and proved that each of these mutations has critical role (Ozturk et al., 2007; Oztürk and Mandaci, 2007; Oztürk and Watmough, 2011). On the other hand, partially conserved residues may also be interesting sites for studies directed at understanding the diversity, mechanism and structure of this highly diverse family.

In this work, 365 different sequences obtained from Heme-Copper Oxidase reductase database (<http://www.evocell.org/hco>) for C-family were aligned; alignments are used to identify partially conserved residues in C-Type oxygen reductases which might play important role in the *cbb₃*-family of oxygen reductases. Sequence alignments showed that Asparagine 265 (N265), Tyrosine 420 (Y420), and Phenylalanine 480 (F480) residues are partially conserved which are unique within the *cbb₃*-type oxidases. In addition to this, there is no published data about these residues has been found to date. In this study, to show the structural and/or functional significance of the partially conserved residues, N265, Y420, and F480, in CcoN subunit of *cbb₃*-type oxidase in *Rhodobacter capsulatus* were substituted for Valine, Phenylalanine and Alanine respectively by site directed mutagenesis. Mutants were characterized with respect to their effects on activity, assembly and proton pumping stoichiometry of the *cbb₃*-type oxidase from *R. capsulatus*.

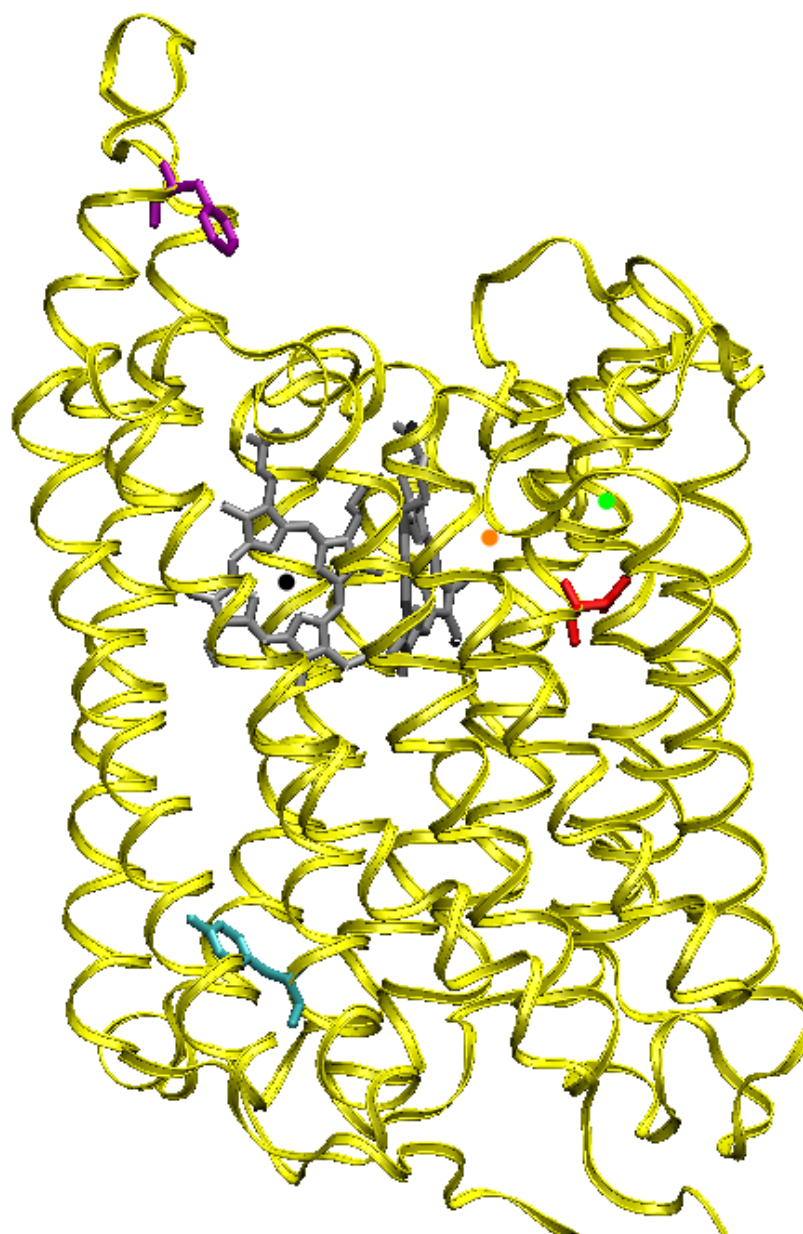


Figure 1.13 Target partially conserved residues on structure of *cbb3*-type oxidase. Structure of *cbb3*-type oxidase is from *Pseudomonas stutzeri*. (PDB ID: 3MK7 (Buschmann et al., 2010)) with CcoN in yellow. Heme groups are in silver, iron ions in black, copper ions in orange, calcium ions in green and target residues, N265, Y420 and F480 are in red, cyan and purple respectively. This figure was generated by using VMD software, version 1.9 (Humphrey et al., 1996).

CHAPTER 2

MATERIALS AND METHODS

2.1. *Bacterial Strains and Growth Conditions*

All *Escherichia coli* and *Rhodobacter capsulatus* strains and plasmids used in this work are listed in Table 2.1. *Escherichia coli* strains and their plasmid-containing derivatives were grown in Luria-Bertani (LB) medium supplemented with appropriate antibiotics (ampicillin, 100 µg/ml; kanamycin, 50 µg/ml; tetracycline, 12.5 µg/ml per ml) for plasmid selection at 37°C on plates or liquids (by shaking 170-175 rpm) as described in (Daldal et al., 1986). *Rhodobacter capsulatus* strains were grown either chemoheterotrophically (Res Growth) or photosynthetically (PS Growth) as appropriate on minimal (MedA) (Sistrom, 1960) or enriched (MPYE) media supplemented with appropriate antibiotics (tetracycline, 2.5 µg/ml; kanamycin, 10 µg/ml and spectinomycin 10 µg/ml) (Daldal et al., 1986) at 35°C with shaking at 100 rpm. All ingredients of the culture media and the preparation of the antibiotic solutions are listed in Appendix A and Appendix B.

Table 2.1 Bacterial strains and plasmids used in this study

Strain	Genotype	Phenotype	Reference
<i>Escherichia coli</i>			
XL1-Blue	rec A end A 1 gyr A986 thi-1hsdr17supE44 rel A1 lac		Stratagene
XL1-Blue/pMOZIN265V		Amp ^r	This work
XL1-Blue/pGY		Amp ^r	This work
XL1-Blue/pGY*Y420F		Amp ^r	This work
XL1-Blue/pGY*F480A		Amp ^r	This work
XL1-Blue/pGYY420F		Amp ^r	This work
XL1-Blue/pGYF480A		Amp ^r	This work
HB101	F-proA2 hsdS20 (Rb-, mB-) recA13 ara14 lac Y1		(Lacks and Greenberg, 1977)
HB101/pMOZI		Amp ^r	(Ozturk et al., 2007; Oztürk and Mandaci, 2007)
HB101/pACYC177	F-ara-14leu fhuA2 Δ(gpt-proA) 62 lac Y1glnV44 galK2 rspL20 xyl-5 mtl-1 Δ(mcrC-mrr) _{HB101}		New England Biolabs
HB101/pMOZII		Amp ^r	(Ozturk et al., 2007; Oztürk and Mandaci, 2007)
HB101/pMOZIIN265V		Amp ^r	This work
DH5α	F- 80dlacZ M15 (lacZYA-argF) U169 recA1 endA1hsdR17(rk-, mk+) phoAsupE44 -thi-1 gyrA96 relA1		(Woodcock et al., 1989)
DH5α /pOX15	15.3 kb with pOX15 DNA	Tet ^r	(Koch et al., 1998)
DH5α /pOX15N265V		Tet ^r	This work
DH5α /pOX15Y420F		Tet ^r	This work
DH5α /pOX15F480A		Tet ^r	This work
TOP10	F ⁻ mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG λ-		Invitrogen
TOP10/pGY2		Amp ^r	This work
TOP10/pGY2ccoNOQP4HIS		Amp ^r	This work
TOP10/pGY2ccoNOQP8HIS		Amp ^r	This work
Top10/pCcoN6HisOQP8His		Tet ^r	This work
<i>Rhodobacter capsulatus</i>			
MT1131	crtD121 Rif ^r	Wild type	(Daldal et al., 1986)
GK32	Δ(ccoNO::kan)	Kan ^r	(Koch et al., 1998)
KZ101	Δ(ccoNO::kan); Δ (cydA::spect)	Kan ^r , Tet ^r , Spect ^r	Provided by Dr. Fevzi Daldal Not published
GK32/pOX15N265V		Tet ^r , Kan ^r	This work
GK32/pOX15Y420F		Tet ^r , Kan ^r	This work

GK32/pOX15F480A	Tet ^r , Kan ^r	This work
GK32/pCcoN-HisOQP	Tet ^r , Kan ^r	Provided by Dr. Grzegorz Pawlik Not published
GK32/pCcoN6HisOQP8His	Tet ^r , Kan ^r	This work
KZ101/pOX15	Tet ^r , Kan ^r , Spect ^r	This work
KZ101/pRK415	Tet ^r , Kan ^r , Spect ^r	This work
KZ101/pOX15F480A	Tet ^r , Kan ^r , Spect ^r	This work

Plasmid	Genotype	Phenotype	Reference
pBluescript	SKII+	Amp ^r	Fermentas
pMOZI	pBluescript SKII with 2.8 kb <i>NO'</i> <i>XhoI</i> insert	Amp ^r	(Ozturk et al., 2007; Oztürk and Mandaci, 2007)
pGY	pBluescript SKII with 2.8 kb <i>ON'</i> <i>XhoI</i> insert	Amp ^r	This work
pGY2	pBluescript SKII with 4.6 kb <i>NOQP'</i> <i>KpnI</i> and <i>Spe I</i> insert	Amp ^r	This work
pGY2 <i>ccoNOQP</i> 4HIS	pGY2 with four histidine codons at C-terminus of <i>ccoP</i>	Amp ^r	This work
pGY2 <i>ccoNOQP</i> 8HIS	pGY2 with eight histidine codons at C-terminus of <i>ccoP</i>	Amp ^r	This work
PMOZIN265V	Substitution of Asparagine to Valine in pMOZI	Amp ^r	This work
pGY*Y420F	Substitution of Tyrosine to Phenylalanine in pGY		
pGY*F480A	Substitution of Phenylalanine to Alanine in pGY		
pACYC177		Amp ^r , Kan ^r	Fermentas
pMOZII	pACYC177 with 2.8 kb <i>NO'</i> <i>XhoI</i> insert	Amp ^r , Kan ^r	(Ozturk et al., 2007; Oztürk and Mandaci, 2007)
pMOZIIN265V	pMOZII replaced 0.6 kb <i>EcoRI</i> and <i>SphI</i> fragment of pMOZIN265V	Amp ^r	This work
pGYY420F	pGY replaced 0.6 kb <i>SacI</i> and I <i>SphI</i> fragment of pGY*Y420F	Amp ^r	This work
pGYF480A	pGY replaced 0.6 kb <i>SacI</i> and I <i>SphI</i> fragment of pGY*F480A	Amp ^r	This work
pOX15	pRK404 with 4.7 kb <i>ccoNOQP</i> operon	Tet ^r	(Koch et al., 1998)
pOX15N265V	pOX15 replaced 2.8 kb <i>XhoI</i> fragment of pMOZIIN265V	Tet ^r	This work
pOX15Y420F	pOX15 replaced 2.8 kb <i>XhoI</i> fragment of pGYY420F	Tet ^r	This work
pOX15F480A	pOX15 replaced 2.8 kb <i>XhoI</i> fragment of pGYF480A	Tet ^r	This work
pOX 12	pOX 15 lacks 2.8 kb <i>NO'</i> fragment (12.5 kb)	Tet ^r	(Ozturk et al., 2007; Oztürk and Mandaci, 2007)
pCcoN-HisOQP	pRK404 with 4.7 kb <i>ccoNOQP</i> operon, six histine codons at C-terminus of <i>ccoN</i>	Tet ^r	Provided by Dr. Grzegorz Pawlik Not published
pCcoN6HisOQP8His	pRK404 with 4.7 kb <i>ccoNOQP</i> operon, six histine codons at C-terminus of <i>ccoN</i> and eight histine codons at C-terminus of <i>ccoP</i>	Tet ^r	This work
pRK2013	self-transmissible plasmid <i>tra</i> ⁺ (RK2)	Kan ^r , helper	(Ditta et al., 1985)

2.2. Plasmid DNA Isolation

A freshly grown single colony of *E. coli* strains were inoculated to 5-10 ml culture of LB medium with suitable antibiotic provided to each sample and grown at 37°C with shaking at 170-175 rpm for 16-18 hours. 5 ml cultures were prepared for the strains containing high copy (pMOZI and pGY) and 10 ml cultures were used for low copy (pMOZII and pOX15) number of plasmids. DNAs were isolated by using two different isolation kits depending on the presence of *endA1* gene in the competent cells. GeneJET™ Plasmid Miniprep Kit (Fermentas cat. #:K0503) and Wizard®*Plus* SV Minipreps DNA Purification System (Promega cat. #: A1330) were used for the isolation of DNAs by following the manufacturer protocols.

2.3. Digestion of Vector and Insert DNAs with Restriction Endonucleases

Insert and plasmid DNAs were cut by using the appropriate endonucleases (Fermentas) according to manufacturers' instructions. The name and catalog number of restriction endonucleases are listed at Appendix E. For cloning, 2 µg plasmid DNAs (from pGY and pMOZI) were digested with 2 µl of restriction enzymes (10 U/µl) in 50-60 µl final volume at 37°C for overnight. For fast digestion, 2 µl plasmid DNA was digested with 1 µl of restriction enzymes (10 U/µl) in 50-60 µl final volume at 37°C for 30 minutes and dephosphorylation was achieved by incubating digested plasmids at 37°C for 45 minutes. As the pRK derivative plasmids are heat sensitive, pOX15 plasmid was digested with only fast digest restriction enzymes at 37°C for 15 minutes and dephosphorylated at 37°C

for 15 minutes. For orientation check of the clones, 200 ng plasmid DNA was digested with 0.5 µl restriction enzyme (5U/µl) in 20 µl final volume at 37°C for 1 hour.

2.4. Isolation of DNA Fragments from Agarose Gel

For the isolation of digested DNA fragments, digestion mixture was loaded on 1% agarose gel and the gel was run for approximately one hour to separate reaction products. Agarose gel bands containing the desired DNA were excised by using QIAquick Gel Extraction Kit (Qiagen cat. #: 28704), Wizard[®] SV Gel and PCR Clean-Up System (Promega cat. #: A9281), Silica Bead DNA Gel Extraction Kit (Fermentas cat. #: K0513) or High Pure PCR Product Purification Kit (Roche cat #: 11732668001) following the manufacturers' instructions.

2.5. Ligation Reactions

T₄ DNALigases from two different manufacturers (Appendix E) were used for the ligation reactions. The concentrations of vector and insert were determined by either spectrophotometry at OD₂₆₀ or comparison of its value with 1 kbp marker on agarose gel. 50- 100 ng vector and 3/1 insert -vector ratio were used for the ligation reactions. The amount of insert was calculated by using the following equation:

$$\text{insert mass in ng} = \text{ratio} \times (\text{insert length in bp} / \text{vector length in bp}) \times \text{vector mass in ng}$$

X ng vector, 3X ng insert, 1.5 µl 10X ligation buffer and 2U-4U T₄ DNA Ligase enzyme were combined and volume was completed to 15 µl with ddH₂O.

While working with pBluescript and pACYC177 derivative vectors, the ligation mixture was incubated at 22°C for 1 hour. On the other hand pRK derivative vector (pOX12) containing ligation mixture was incubated at 22°C for 1 hour than transferred to 8°C and incubated for 16 hours. After ligation process, T₄ DNA Ligase was inactivated by incubation at 65°C for 10 minutes.

2.6. Competent Cell Preparation

2.6.1. HB101 and XL1-Blue Competent Cell Preparation and CaCl₂ Transformation

For *E.coli* HB101 and XL1-Blue competent cells, 50 ml LB in a 500 ml flask was inoculated with 500 µl overnight culture of *E.coli* HB101 and XL1-Blue strains. The inoculated cultures were incubated at 37°C with vigorous shaking about 2 hours until OD₆₀₀ reaches to 0.4-0.5, after cells reached to Log phase they were taken on ice and transferred to ice cold Falcon tubes. 40 ml cells were centrifuged at 5000 rpm for 10 minutes at 4°C. Supernatant was discarded and pellet was resuspended in 20 ml (1/2 of the culture volume) of ice cold 100mM CaCl₂ and incubated on ice for 30 minutes. After incubation period, cells centrifuged at 5000 rpm for 10 minutes at 4°C. Supernatant was discarded carefully and pellet was resuspended in 2 ml (1/20 of the culture volume) of ice cold 100mM CaCl₂ and glycerol (10% of final concentration). Competent cells were aliquoted as 100 µl into sterile Eppendorf tubes and stored at -80°C.

For CaCl₂ transformation, 10 µl ligation reaction mixture was added to 100 µl competent cells and incubated on ice for 45 minutes. Then heat shock was applied to the cells by incubating them at 37°C for 5 minutes. Samples were re-

taken on ice and incubated for 5 minutes. 1 ml LB was added to the samples and cells were incubated for 1 hour at 37°C with vigorous shaking. After incubation, cells were centrifuged at 13.000 rpm for 1 min at RT and pellet was resuspended in 100 µl LB. Cells were plated on selection plates and incubated at 37°C for 16-18 hours (Davis et al., 1988).

2.6.2. DH5α Competent Cell Preparation and Transformation

For *E.coli* DH5α competent cells, 50 ml SOB (Appendix A) in a 500 ml flask was inoculated with 2-3 colonies of overnight culture of *E.coli* DH5α strains. The inoculated cultures were incubated at RT with shaking approximately 20 hours until OD₆₀₀ reaches 0.5-0.6. After cells grown to expected OD, they were transferred to ice cold Falcon tubes and incubated for 10 minutes. 40 ml cells were centrifuged at 2500 *g* for 10 minutes at 4 °C. Supernatant was discarded and pellet was resuspended in 12.8 ml of ice cold Transformation Buffer (TB) (Appendix C) and incubated on ice for 10 minutes. Cells centrifuged at 2500 *g* for 10 minutes at 4°C. Supernatant was discarded and pellet was resuspended in 3.2 ml of ice cold TB and 224 µl sterile DMSO (7% of final concentration). Cells were incubated on ice for 10 minutes and then competent cells were aliquoted as 200 µl into sterile Eppendorf tubes and stored at -80°C for further use.

For transformation, *E. coli* DH5α competent cells were thawed at RT and 5 µl DNA was added to 200 µl competent cells and incubated on ice for 30 minutes. Then heat shock applied at 42°C for 1 minute to the cells. Samples were re-taken on ice and incubated for 5 minutes. 800 µl SOC was added to the samples and cells were incubated at 37°C for 1 hour with shaking. After incubation, cells were

centrifuged at 4.000 rpm for 5 min at RT and pellet was resuspended in 100 µl SOC. Cells were plated on selection plates and incubated at 37°C for 16-18 hours.

2.7. Construction of pGY for Y420 and F480 Mutations

For construction of pGY DNA, pMOZIDNA (Oztürk and Mandaci, 2007) was isolated by using GeneJET™ Plasmid Miniprep Kit (Fermentas) and digested with *Xho*I Fast Digest (Appendix E) restriction enzyme for 30 minutes at 37°C. The resulting fragments were determined on agarose gel and ethanol precipitation was done to *Xho*I digested pMOZI DNA for removing the digestion mixture components. *Xho*I digested pMOZI fragments were used as vector and insert for the ligation reaction during 1 hour at 22°C. T₄ DNALigase was inactivated by heating reaction mixture at 65°C for 10 minutes. 10 µl ligation reactions was transformed to CaCl₂ treated XL1-Blue competent cells and plated on LB plates containing IPTG, X-Gal and ampicillin. Transformants were selected by using Blue/White Colony Assay in the presence of ampicillin (100µg/ml).

To select the correctly oriented insert, plasmid DNAs were isolated from transformants and digested with *Sph*I (Appendix E) and *Sac*I (Appendix E) restriction enzymes at 37°C ON. *Sph*I and *Sac*I enzymes cut plasmid DNA in two different ways, if insert is correctly oriented (targeted new template DNA), 5.1 kbp and 0.6 kbp fragments are produced, on the other hand if insert is wrong oriented (pMOZI), 3.2 kbp and 2.5 kbp fragments are produced after digestion. The new clone which has correct orientation was designated as XL1-Blue/pGY.

2.8. PCR-Based Site-Directed Mutagenesis

The Quick Change Site-Directed Mutagenesis (Stratagene) Protocol was used to create mutations. In this method, *Pfu* DNA polymerase (Appendix E) which has lower error rate compared to the other polymerases and avoids second site-mutations (Flaman et al., 1994; Cline et al., 1996) was used as polymerase enzyme and PCR reactions were performed by using thermal temperature cycler (Techne TC-3000 (Appendix F). In the procedure of the site-directed mutagenesis, pMOZI DNA was used as a template for the substitution of Asparagine 265 for Valine 265 and pGY DNA was used as template for the substitution of Tyrosine 420 for Phenylalanine 420 and Phenylalanine 480 for Alanine 480 by using forward and reverse primers for each mutations. Forward and reverse primers designed on nucleotide sequence of subunit I of *cbb₃* oxidase in *R. capsulatus* (Table 2.2 and 2.3). PCR reactions were prepared as defined on Table 2.4 for each mutation, *Pfu* polymerase enzyme was not used in control reaction. All reaction mixtures were incubated at the conditions shown in Table 2.5. After PCR reactions 10 µl of PCR products of each mutation and control reaction were loaded on 1% agarose gel and visualization of DNAs was performed by electrophoresis for 1 hour.

Following the PCR reactions, products were treated with *DpnI* (Appendix E) for 2 hours at 37°C to digest the parental template DNA and to select the PCR synthesized DNA. After PCR product digestion, *DpnI* treated PCR products and untreated PCR products and controls were loaded on 0.8 % agarose gel and electrophoresed for 1 hour. After visualization PCR products and controls, untreated PCR products and controls were transformed into XL-1 blue cells.

Finally, to find transformants with desired mutations, four transformants for each mutation were selected randomly and DNA isolation was performed for sequencing.

Table 2.2 Nucleotide sequence of subunit I of the *cbb₃*-type oxidase in *R. capsulatus*^a

>gi 294675557:1226693-1228291 Rhodobacter capsulatus SB 1003 chromosome, from complete genome	
ATGTGGGATTACGTCAAGCTTGTGCGCTTGGCGTCGTGGTGGCTATTGCCGCTTACGCC	60
GCCAGTCAGGCGCGAGATTTGCCGTATATGGTGAACATGGTCGAGGTCGCGCTGGCGGCC	120
GTGATCGCGTTTCATCTGGGTGCTGCGCACCATGGGGGACGCCAAACCCTCGAAAGACGAG	180
TATTTTCGACGGCGTGATCCGCGCCGGCGTGATCGCAACCACCTTCTGGGGGATCGTCGGC	240
TTTCTCGTGGCCGTATCATCGCCTTCCAGCTTGCCTTCCCCGCGCTGAACCTTGAATTC	300
GGCAACGGGATGCTGAACTTCGGGCGCCTGCGTCCGCTGCACACCTCGGCGGTGATCTTC	360
GCCTTTGGCGGCAACGCCCTGATCGCCTCGGCCTTCTATGTCGTGCAGCGCACCTCGGCG	420
GCGCGTCTCTTCGGGGGCACGGCGCTTGGCTGGTTCTGTGTTCTGGGGCTGGCAGCTGATC	480
ATCGTCAACGCCGCGACCTCCTATCTGCTGGGCGGCTCGCAGGGCAAGGAATATGCCGAG	540
CTGAACTGGCATCTCGACATCCTCGTCGCGATCGTCTGGGTGGCCTATCTGATCGCCTTC	600
CTCGGCACGATCTTCAAGCGCAAGGAACCCACATCTACGTGGCGAACTGGTTCTACCTG	660
TCCTTCATCGTGACGATCGCCATGTTGCACATCGTCAACAACCTGGCCGTTCCGGTCTCG	720
ATCTTCGGCACCAAATCGGTGCAGCTGATGGCGGGCGTGCAAGGATGCGATGACGCAATGG	780
TGGTACGGCCAC AAC GCCGTGGGCTTCTTCCTACCGCGGGCTTTCTGGGCATGATGTAT	840
TACTTCGTGCCGAAACAGGCCGAGCGTCCCGTCTATTCTACAAGCTCTCGATCGTCCAC	900
TTCTGGGCGCTGATCTTCCTCTACATCTGGGCCGGTCCGCACCACCTGCATTATACCGCG	960
CTGCCCCACTGGGCCTCGACGCTGGGCATGGTGATGTCGGTGATCCTGTGGATGCCGTCC	1020
TGGGGCGGCATGATCAACGGCCTGATGACGCTTTTCGGGCGCCTGGGACAAGCTGCGCACC	1080
GATCCGGTGATCCGGATGATGGTGGTTTCGATCGGCTTTTACGGCATGTCGACCTTTGAA	1140
GGCCCGATGATGTCGATCAAGGCGGTCAACTCGCTCTCGCACTACACGGACTGGACCATC	1200
GGTCACGTGCATTTCGGGCGCGCTGGGCTGGAACGGCATGATCACCTTCGGCATGCTC TAC	1260
TTCTTGACGCCGCGCCTGTGGGGCCGTTTCGGGGCTTTACTCGCTGAAACTGGTCAGCTGG	1320
CACTTCTGGCTCGCCACCATCGGGATCGTGCTTTACGCCTCCTCGATGTGGGTGTCGGGC	1380
ATCATGGAAGGCCTGATGTGGCGTGAAGTCGATGCGCAGGGCTTCCTGGTGAACGGT TTC	1440
GCCGACACCGTGGGCGCCAAGTTCCCGATGAACGTCGTGCGTGCGTCGGGGGTGTGCTG	1500
TATCTGACCGGCGGTCTGATCATGGCCTACAACCTTTGGGCCACGGTCGCGAAACAACCG	1560
AAGACGGCCAATCTCGCCGTTGCGGTTCCGGCGGAATAA	1599

^aCodons of the target conserved amino acids, N265, Y420, and F480 respectively were highlighted and labeled

Table 2.3 Forward and reverse primers used for the site directed mutagenesis

Primers	Sequence of the primers
N265VF	5'-GGTGGTACGGCCAC <u>GTG</u> GCCGTGGG-3'
N265VR	5'-CCCACGGCC <u>CAC</u> GTGGCCGTACCACC-3'
Y420FF	5'-CCTTCGGCATGCTC <u>TTC</u> TCCTGACGCCGC-3'
Y420FR	5'-GCGGCGTCAGGAAG <u>AAG</u> AGCATGCCGAAGG-3'
F480AF	5'-CCTGGTGAACGGT <u>GCC</u> GCCGACACCGTGGGC-3'
F480AR	5'-GCCCACGGTGTCGG <u>CGG</u> CACCGTTCACCAGG-3'

Table 2.4 PCR reactions for site- directed mutagenesis

Reactants	Sample Reaction	Control Reaction
Template DNA (10 ng/μl)	5 μl	5 μl
Buffer (10X <i>Pfu</i> polymerase buffer)	5 μl	5 μl
Forward Primer (125 ng/μl)	1 μl	1 μl
Reverse Primer (125 ng/μl)	1 μl	1 μl
dNTP mix (10mM)	1 μl	1 μl
<i>Pfu</i> polymerase (5u/ μl)	1 μl	-
ddH ₂ O	36 μl	37 μl
Total reaction volume	50 μl	50 μl

Table 2.5 PCR conditions for site-directed mutagenesis

Cycle	Temperature	Time
First denaturation	95 °C	30 sec
Denaturation	95 °C	30 sec
Annealing	65 °C/68°C	1 min
Extension	72 °C	15 min
Number of cycles	16	
Final hold	4 °C	-

2.9. DNA Sequencing

To see the desired mutations, pMOZIN265V and pGY*Y420F and pGY*F480A DNAs were partially sequenced (0.9 kbp *EcoRI* and *SphI* fragment of pMOZI DNA and 0.6kbp *SacI* and *SphI* fragment of pGY DNA) by Iontek (İstanbul-Turkey) using primers 568F (5'- CGATCGTCTGGGTGGCC-3'; 17-bp), 214 F (5'- GCAACCACCTTCTGG-3'; 15-bp) and universal primer M13R-PUC (5'-CAGGAAACAGCTATGAC-3'; 17-bp).

2.10. Replacement of the 0.9 kbp NO' Fragment of pMOZII with that of pMOZIN265V Mutant

To replace the mutated 0.9 kbp fragment from pMOZIN265V with pMOZII(Oztürk and Mandaci, 2007), pMOZIN265V and pMOZII DNAs were isolated by using Wizard[®]Plus SV Minipreps DNA Purification System (Promega). Both of the isolated DNAs were digested with *SphI* and *EcoRI* (Appendix E) restriction enzymes at 37°C for overnight (Figure 2.1) and(Figure 2.2). After digestion, fragments were separated on the 1% agarose gel and 0.9 kbp fragments from pMOZIN265V and 5.9 kbp fragment from pMOZII were isolated from agarose gel by using QIAquick Gel Extraction Kit (Qiagen). The 0.9 kbp mutated fragment was inserted into 5.9 kbp vector by ligation process at 22°C for 1hour. After ligation process, 10 µl ligation mixtures were transformed into HB101 competent cells. Transformants were selected on the basis of their size (6.8 kbp) and ampicillin resistance. The new clones which have the desired mutations were saved and designated as HB101/pMOZIIN265V (Figure 2.3).

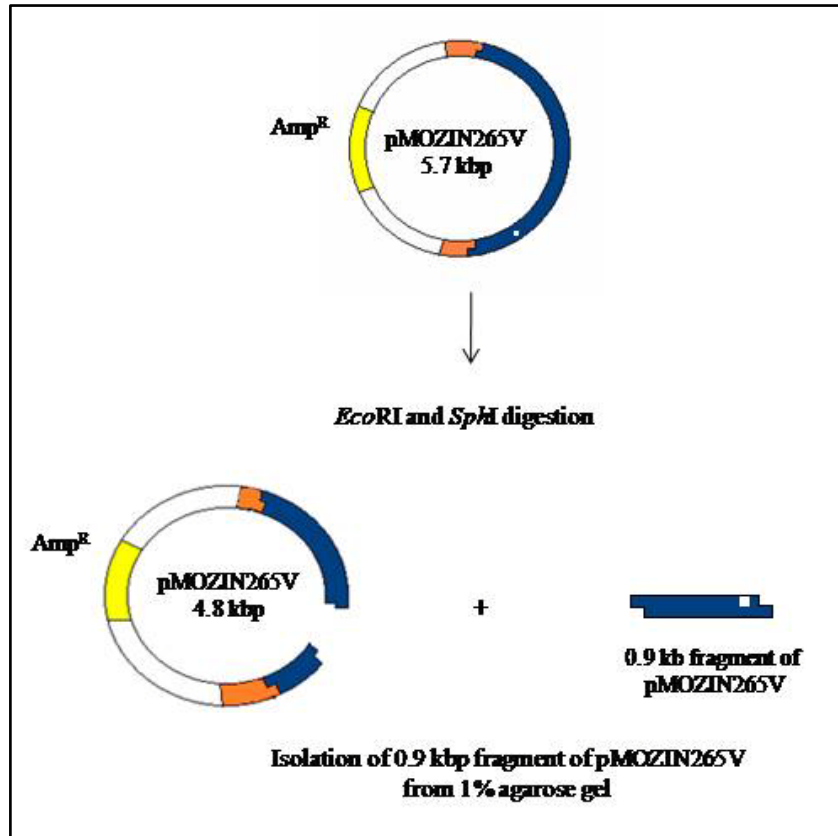


Figure 2.1 Digestion of pMOZIN265V with *EcoRI* and *SphI*

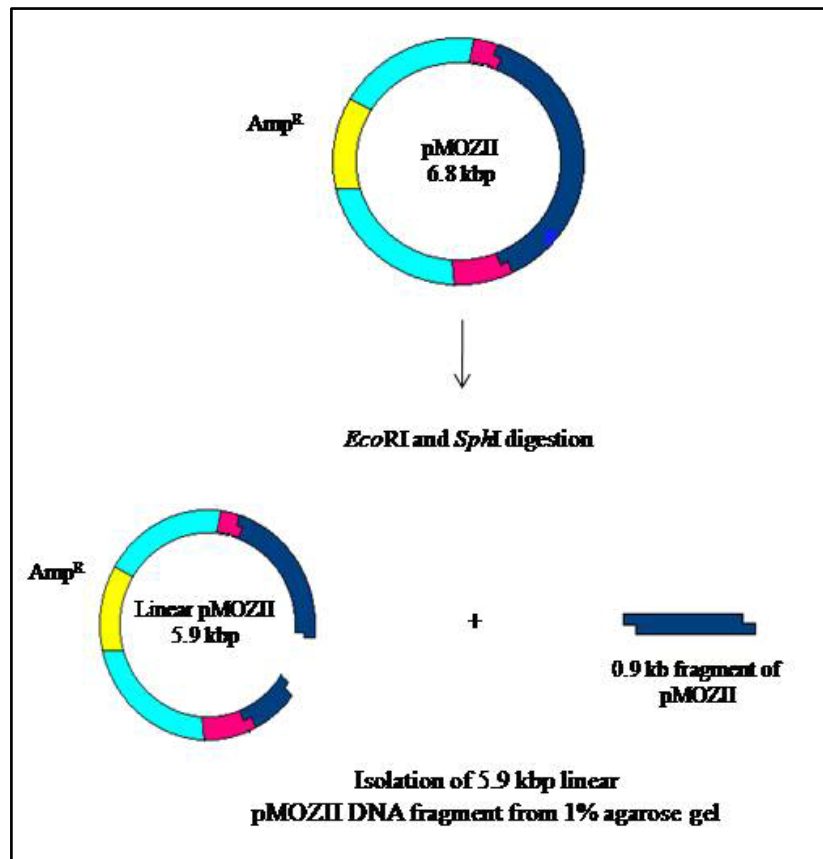


Figure 2.2 Digestion of pMOZII with *Eco*RI and *Sph*I

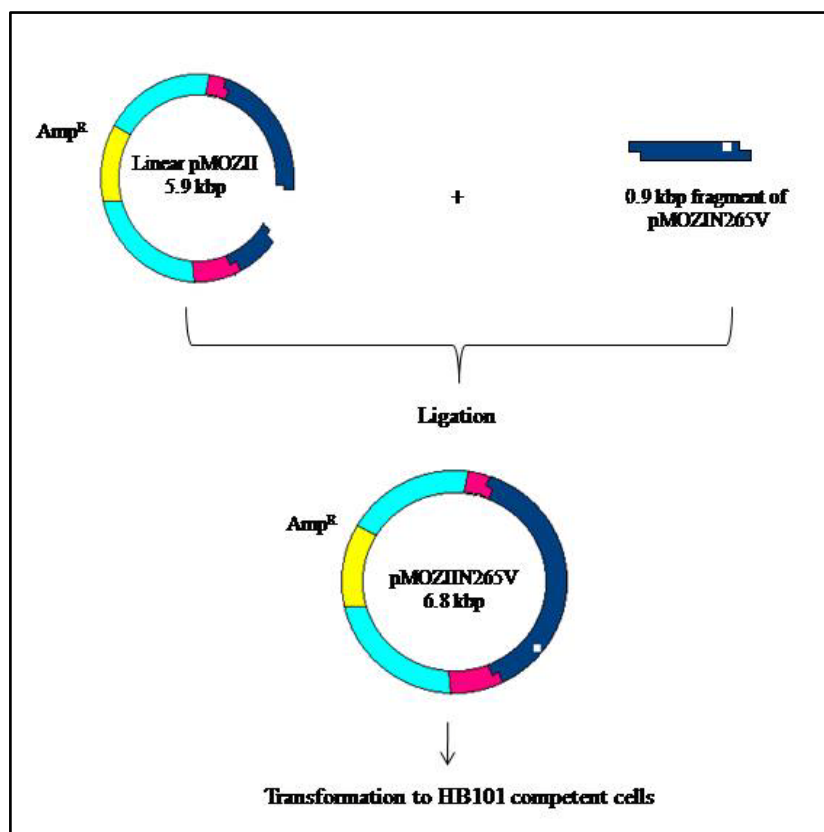


Figure 2.3 Replacement of the 0.9 kbp pMOZII fragment with that of pMOZIN265V

2.11. Replacement of the 0.6 kbp NO' Fragment of pGY with that of pGY*Y420F and pGY*F480A Mutants

Since the sequencing results verify that mutations were created successfully and no unwanted mutations has not been occurred during PCR process, pGY*Y420F and pGY*F480A and pGY DNAs were isolated by using GeneJET™ Plasmid Miniprep Kit (Fermentas) for the replacement of mutated 0.6 kbp fragments with the that of pGY fragment. Isolated DNAs were digested with *Sph*I and *Sac*I restriction enzymes at 37°C for overnight (Figure 2.4). *Sph*I and *Sac*I digested vector (pGY) was dephosphorylated by shrimp alkaline phosphatase(SAP) (Appendix E) to prevent self-ligation of the vector at 37°C for 30 minutes (Figure 2.5). Than the fragments were separated on the 1% agarose

gel and 0.6 kbp mutated fragments from pGY*Y420F and pGY*F480A and 5.1 kbp fragment from pGY were isolated from agarose gel by using QIAquick Gel Extraction Kit (Qiagen). Each 0.6 kbp mutated fragment was inserted into 5.1 kbp vector during ligation process at 22°C for 1hour. 10 µl ligation mixtures were transformed into XL1-Blue competent cells. Transformants were selected on the basis of their size (5.7 kbp). The new clones which have the desired mutations were saved and designated as XL1-Blue/pGYY420F and XL1-Blue/pGYF480A (Figure 2.6). To ensure that each plasmid carries the desired mutations pGYY420F and pGYF480A DNAs were isolated and sequenced.

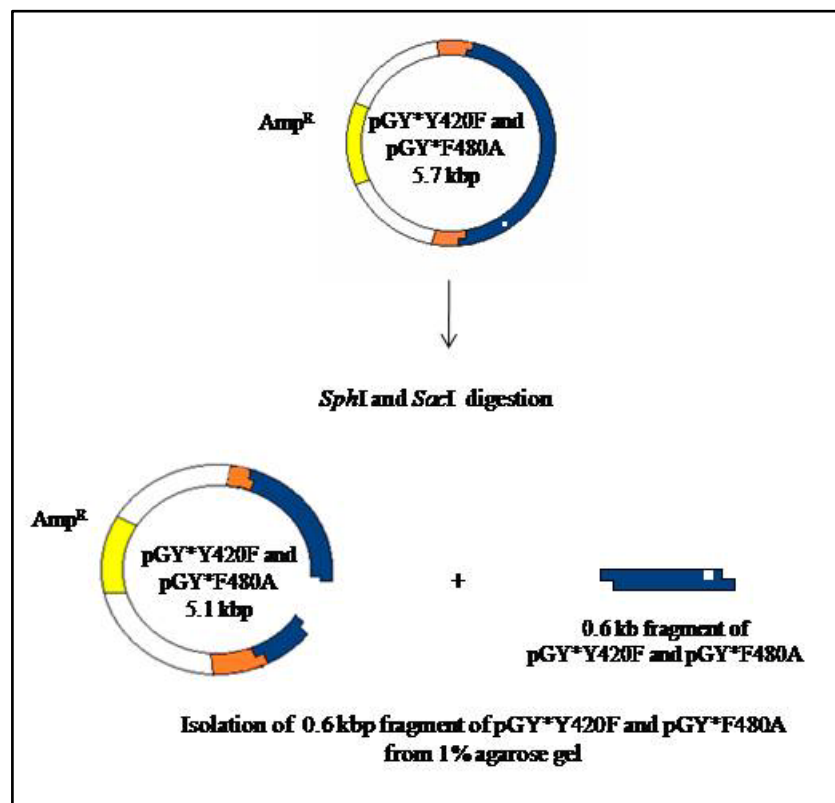


Figure 2.4 Digestion of pGY*Y420F and pGY*F480A with *SphI* and *SacI*

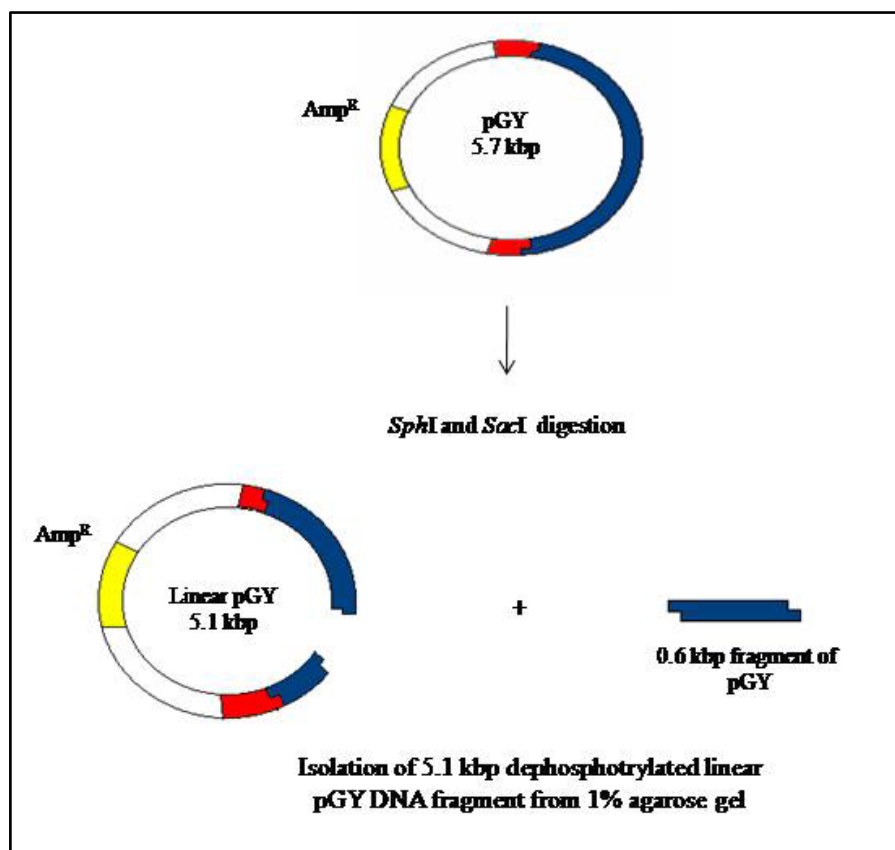


Figure 2.5 Digestion of pGY with *SphI* and *SacI*

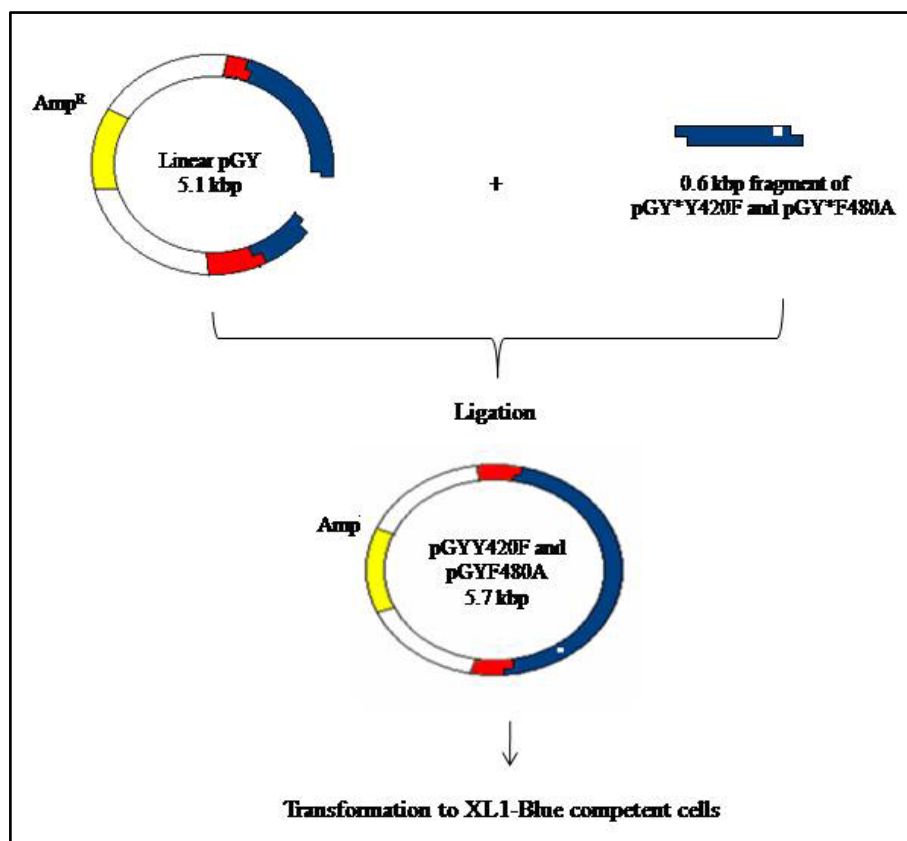


Figure 2.6 Replacement of the 0.6 kbp pGY fragment with that of pGY*Y420F and pGY*F480A

2.12. Replacement of the 2.8 kbp NO' Fragment of pOX15 with that of pMOZIIN265V Mutant

To replace the 2.8 kbp fragment of pOX15 with the 2.8 kbp mutated insert that contains N265V mutation, pMOZIIN265V plasmid DNA was digested with *XhoI* restriction enzyme for 1.5 hours at 37°C. Resulting fragments 4.0 kbp and 2.8 kbp were obtained after digestion (Figure 2.7). On the other hand pOX12 DNA (12.5 kbp) (Oztürk and Mandaci, 2007; Ozturk et al., 2007) was isolated by using Wizard[®] Plus SV Minipreps DNA Purification System (Promega) and digested with *XhoI* restriction enzyme for 1 hour at 37°C and also dephosphorylated by shrimp alkaline phosphatase (SAP) at 37°C for 15 minutes to prevent self-ligation of the vector (Figure 2.8). Then the fragments were

separated on the 1% agarose gel and 2.8 kbp mutated fragment from pMOZIIN265V and 12.5 kbp linear fragments from pOX12 were isolated from agarose gel by using QIAquick Gel Extraction Kit (Qiagen). The 2.8 kbp mutated fragment was inserted into 12.5 kbp vector by ligation process at 22°C for 1 hour than transferred to 8°C and incubated for 16 hours for the completion of the ligation process. After ligation, 10 µl ligation mixtures were transformed into DH5α competent cells and four transformants were selected on the basis of their size (15.3 kbp) and tetracycline resistance (Figure 2.9). Selected transformants were named as DH5α /pOX15*N265V.

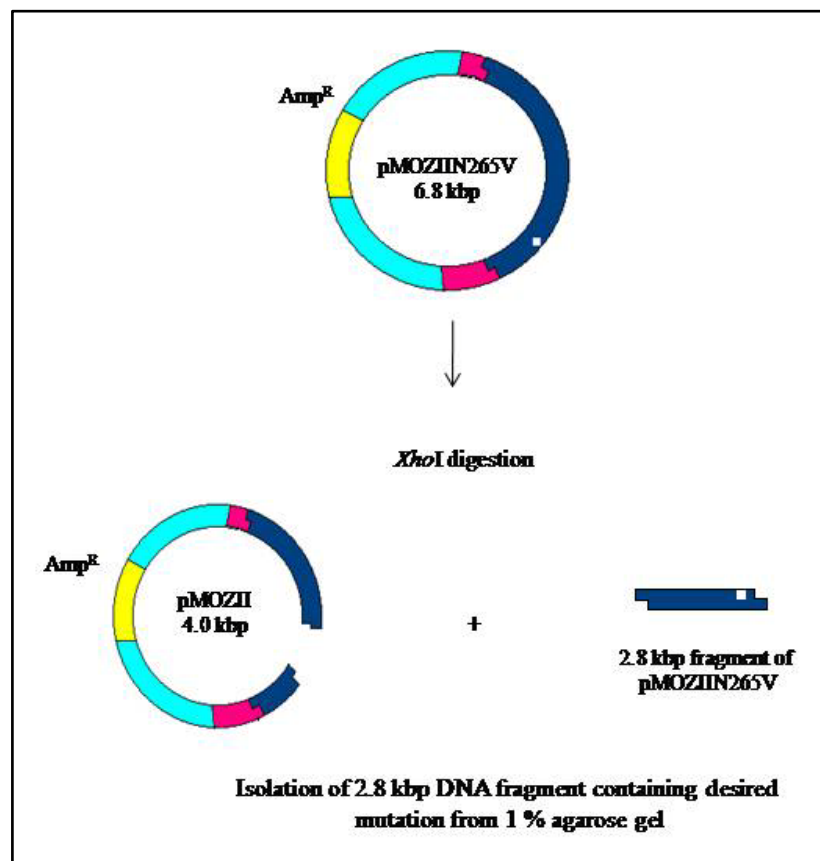


Figure 2.7 Digestion of pMOZIIN265V with XhoI restriction enzyme

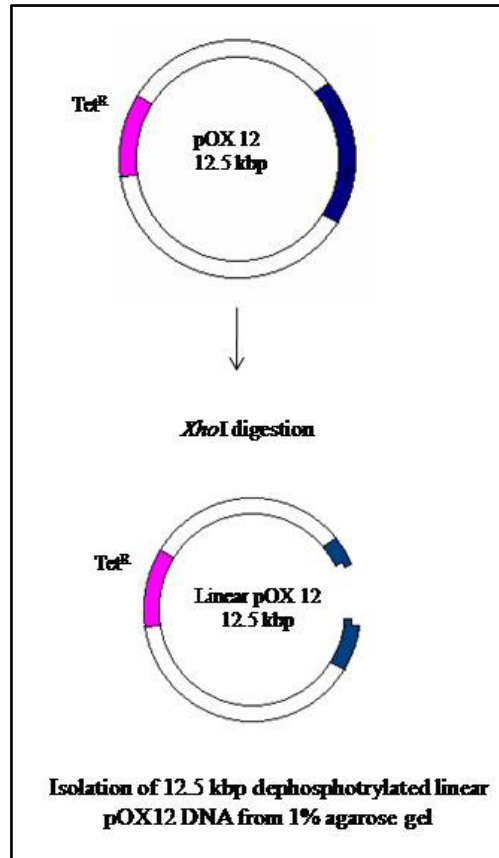


Figure 2.8 Digestion of pOX12 with *Xho*I restriction enzyme

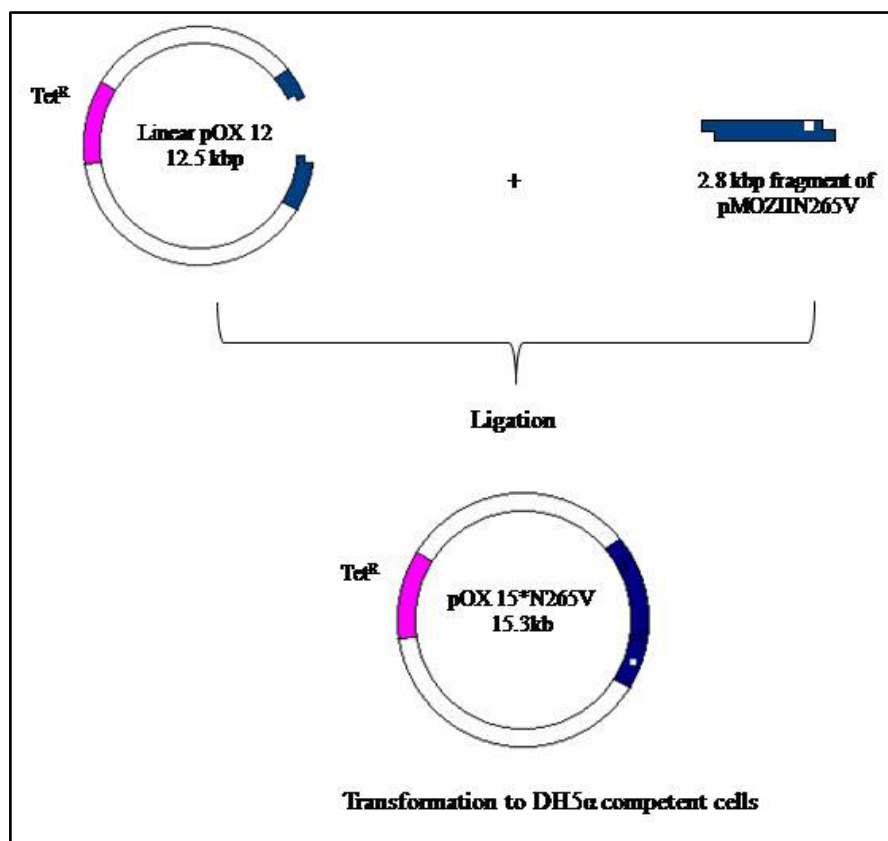


Figure 2.9 Replacement of the 2.8 kbp parental fragment of pOX15 with that of the pMOZIIN265V

2.13. Replacement of the 2.8 kbp NO' Fragment of pOX15 with that of pGYY420F and pGYF480A Mutants

To replace the 2.8 kbp fragment of pOX15 with the 2.8 kbp mutated insert that contains Y420 and F480 mutations, plasmid DNAs from pGYY420F and pGYF480A were isolated and digested with *Alw441* (Appendix E) and *XhoI* for 4 hours at 37 °C. After digestion, 2.8 kbp, 1.3 kbp, 0.9 kbp and 0.7 kbp fragments were obtained (Figure 2.10). On the other hand pOX12 vector was digested and dephosphorylated as described in section 2.12. Following digestion, fragments were separated on the 1% agarose gel and 2.8 kbp mutated fragments from pGYY420F, pGYF480A and 12.5 kbp linear fragment from pOX12 were isolated from agarose gel by using QIAquick Gel Extraction Kit (Qiagen). The 2.8 kbp

mutated fragment was inserted into 12.5 kbp vector by ligation process at 22°C for 1 hour than transferred to 8°C and incubated for 16 hours for the completion of the ligation process. After ligation process, 10 µl ligation mixtures were transformed into DH5α competent cells and transformants were selected on the basis of their size (15.3 kbp) and tetracycline resistance. Selected transformants were named as DH5α /pOX15*Y420F and DH5α /pOX15*F480A.

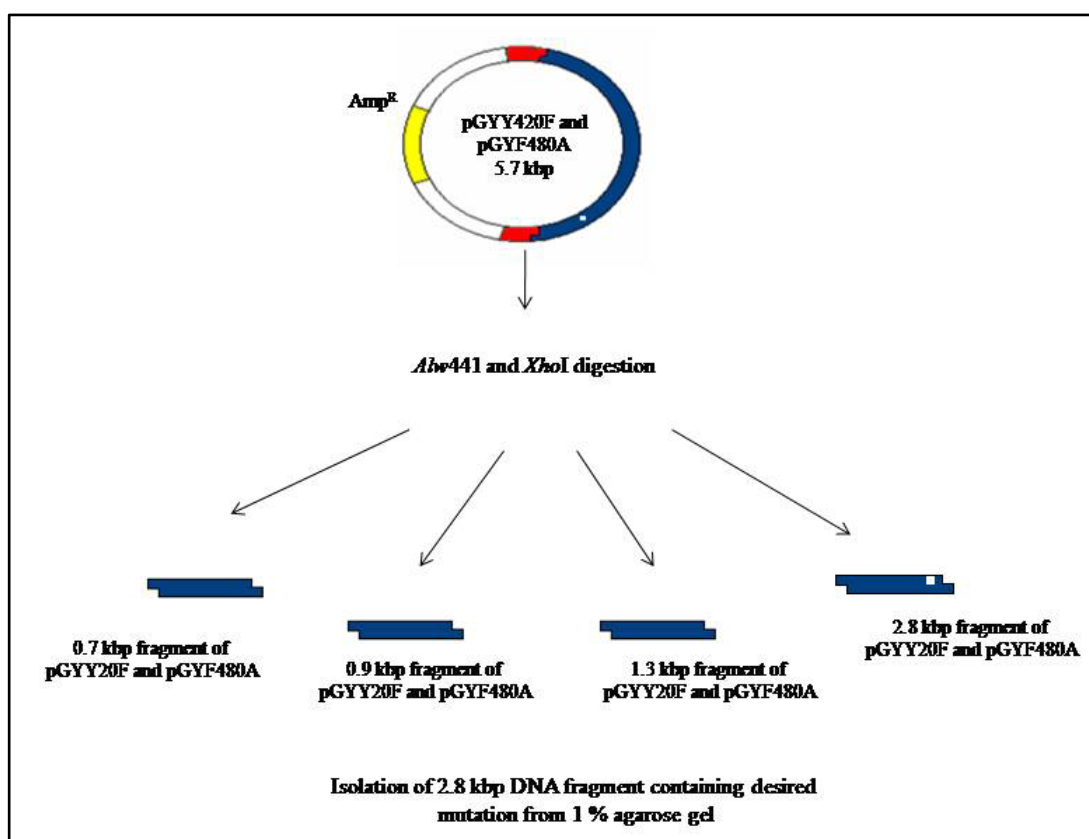


Figure 2.10 Digestion of pGYY420F and pGYF480A with *XhoI* and *Alw441* restriction enzymes

2.14. Control of the 2.8 kbp Insert Orientation

To select the pOX15* clones that have the correct oriented insert, plasmid DNAs were isolated from transformants and digested with *Hind*III (Appendix E) restriction enzyme at 37°C for 1 hour. *Hind*III enzyme cuts plasmid DNA into 11.7 kbp and 3.6 kbp DNA fragments if insert is correctly oriented but 12.5 kbp and 2.8 kbp DNA fragments are produced if insert has wrong orientation (Figure 2.11). The new clones each of which contains one of the three desired mutations were saved and named as DH5 α /pOX15N265V, DH5 α /pOX15Y420F and DH5 α /pOX15F480A.

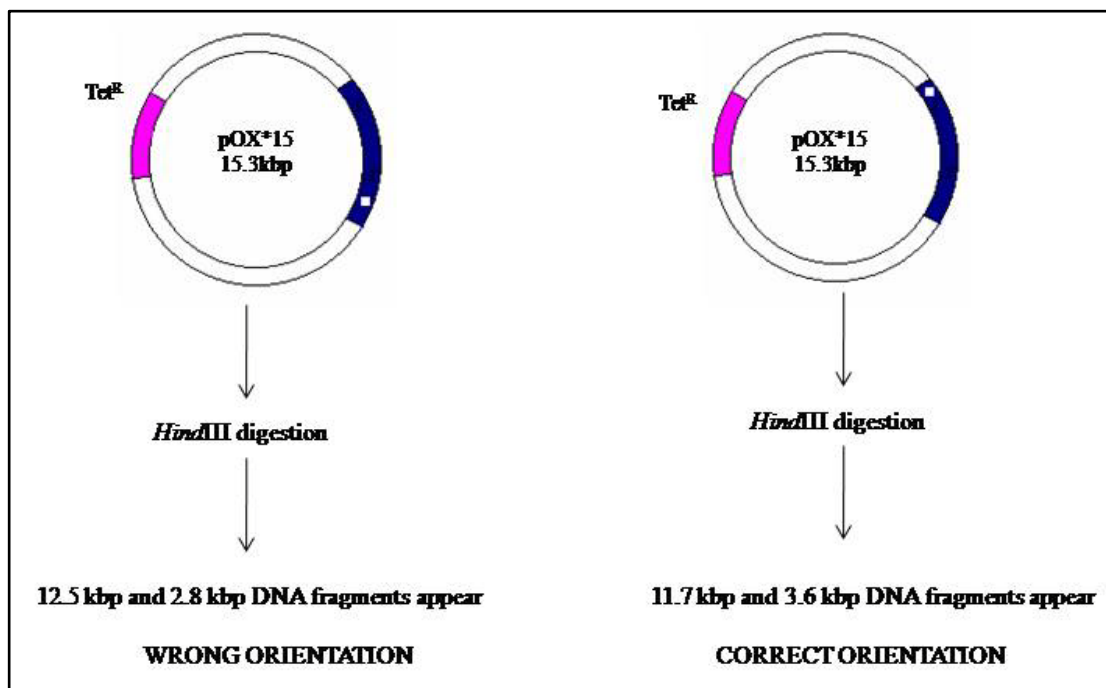


Figure 2.11 Digestion of pOX15* with *Hind*III for orientation control

2.15. Conjugation (Triparental Mating)

As the size of the plasmid (pOX15-15.3 kbp) is a handicap for using other transfer methods, conjugation is the most suitable method for introducing plasmid DNA into *R. capsulatus* cells. In triparental mating, the transfer functions are provided by helper strain, pRK2013, carrying the *tra* genes of RK2 which are used for the transfer of the mobilizable plasmid from separate donor, pOX15, to recipient strain, *R. capsulatus*.

R. capsulatus GK32 strain, lacking *ccoNOQP* gene, complemented with three recombinant plasmids that contain desired mutations by following Triparental Mating procedure described by (Ditta et al., 1985) (Figure 2.13). Two days before the cross, 1 ml Med A medium supplemented with kanamycin was inoculated with a few colonies of the recipient *R. capsulatus* GK32 strain, from a fresh plate and grown 18-24 hours at 35°C with 90 rpm shaking. The day before the cross 1 ml culture was transferred to 10 ml Med A medium supplemented with kanamycin and cells were grown 18-24 hours at 35°C with 90 rpm shaking. The day before the cross, 5 ml LB medium supplemented with kanamycin culture was inoculated with a single colony of the helper strain HB101/pRK2013 from a fresh plate. 10 ml LB medium supplemented with tetracycline was inoculated with the donor strains (DH5 α /pOX15N265V, DH5 α /pOX15Y420F and DH5 α /pOX15F480A) that carries each the mutation from a single colony on a fresh plate and cells were grown for 12-16 hours at 37°C with 175 rpm shaking. After incubation period, cells were centrifuged at 4000 rpm for 10 minutes at 25°C. The supernatant was discarded and pellet was resuspended in 1 ml Med A

medium. The cell density of each culture was determined by using the following conversions:

R. capsulatus: 7.5×10^8 cells/ml at $OD_{630} = 1$

E. coli: 5.0×10^8 cells/ml at $OD_{600} = 1$

During this time period, the required numbers of MedA plates were dried in the incubator for 30 min. After the determination of cell density, the cells were washed twice (10.000 rpm for 3 minutes at 25°C) with 1 ml Med A medium to remove all antibiotics. The cells were resuspended in a volume of Med A medium that the final density was 10^{10} /ml. Then, in the middle of Med A plate, 100 µl of recipient *R. capsulatus* strain (GK32), 20 µg of each of helper (HB101/pRK2013) and donor strains (DH5α/pOX15N265V, DH5α/pOX15Y420F and DH5α/pOX15F480A) were added and mixed well by elbow of bent glass rod. Plates were waited for the absorption of the spot into the agar completely before incubation for 24 hours at 35°C. The positive control plate was prepared with recipient/helper/DH5α/pOX15 and negative control plates include just the recipient/helper and recipient/donor spots. After incubation period, 1ml MedA medium was pipetted on the each spot and by using bent glass rod and tipping the plate cells were scraped off the agar surface into the MedA broth and collected at the one edge of the plate and transferred into eppendorf tubes. Then, the cells were centrifuged for 1 minute at 10.000 rpm at 25°C. The supernatant was discarded, and the cells were washed once in 1 ml Med A medium. Washed cells were resuspended in 400 µl Med A medium. 2 µl, 3 µl and 50 µl of the cell suspension were plated on Med A plates with appropriate antibiotics and incubated for 2-3 nights. At least 4 transconjugants from each cross were selected

on the basis of tetracycline resistance and designated as GK32/pOX15N265V, GK32/pOX15Y420F and GK32/pOX15F480A and stored at -80°C for further use.

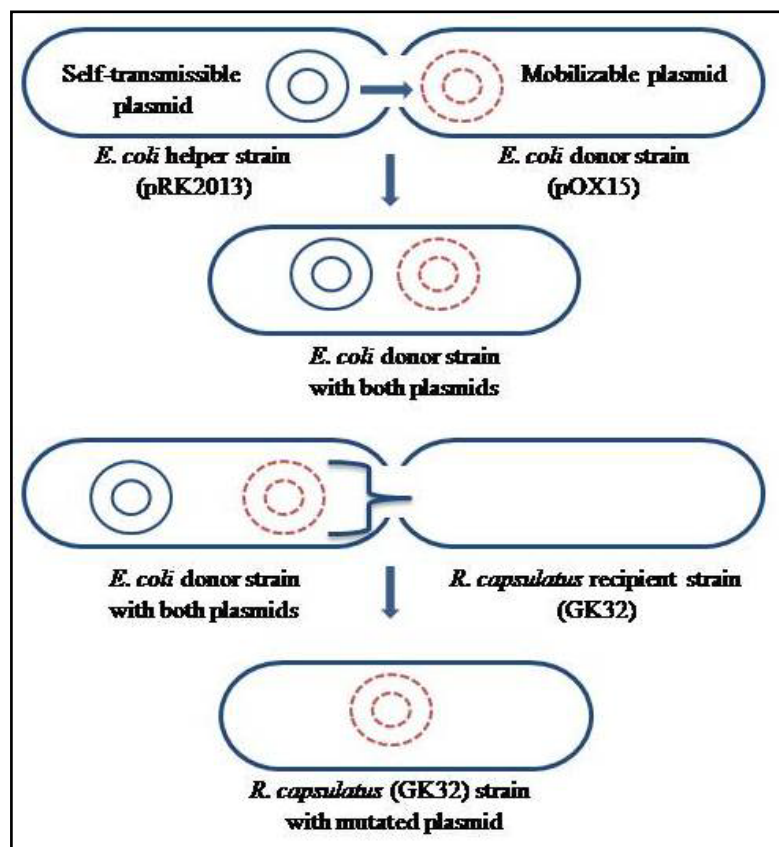


Figure 2.12 Diagram depicting triparental mating

2.16. Analysis of the Cytochrome *cbb*₃ Oxidase Activity and Assembly

2.16.1. NADI Staining

Quantitative analysis of cyt *cbb*₃ oxidase activity was performed by NADI (α -Naphthol + DMPD + O₂ → Indophenol Blue + H₂O) reaction (Keilin, 1966). The presence of only one-type of cyt *c* oxidase in *R. capsulatus* allows the identification of the effects of mutation on cyt *cbb*₃ oxidase activity. All mutagenized colonies (GK32/pOX15N265V, GK32/pOX15Y420F and

GK32/pOX15F480A) with positive (GK32/pOX15) and negative (GK32) controls were tested with NADI reaction by being overlaid with a 1:1 (vol/vol) mixture of 35 mM α -naphthol in ethanol and 30 mM *N,N*-dimethyl-*p*-phenylenediamine in H₂O (Keilin, 1966). Under these conditions, colonies grown on Med A medium turn blue within 30 seconds (NADI⁺ phenotype), if they contain an active cytochrome *cbb*₃ oxidase.

2.16.2. Sequence Analysis

After complementation test with NADI staining, plasmid DNAs from each mutant GK32/pOX15N265V, GK32/pOX15Y420F, GK32/pOX15F480A were isolated by using QIAprep® Miniprep Kit and sequenced by DNA Sequencing Facility of University of Pennsylvania with primers N4 (5'-CCAAATCGGTGCAGCTGATG-3'; 20kbp) and N5 (5'-CGATCAAGGCGGTCAACTCG3'; 20kbp) to guarantee that each plasmid carries the target mutations before starting characterization experiments.

2.16.3. Preparation of Chromatophore Membrane Proteins

The chromatophore membranes proteins (intracytoplasmic membrane vesicles-ICM) of MT1131, GK32, GK32/pOX15, GK32/pOX15N265V, GK32/pOX15Y420F and GK32/pOX15F480A were prepared according to (Gray et al., 1994).

Three days before the chromatophore membrane protein isolation, 1ml MPYE medium supplemented with tetracycline was inoculated with a single colony of MT1131, GK32 and mutant strains of *R. capsulatus* from a freshly

streaked plate and were grown ON (18-24hrs) at 34°C in dark with shaking at 150 rpm. Next day, 1ml culture was transferred to 10ml MPYE medium supplemented with tetracycline and cells were grown ON until $OD_{630} \geq 0.6$ at 34°C in dark with shaking at 150 rpm. The day before starting isolation, 10 ml cultures was transferred to 1L MPYE medium supplemented with tetracycline in 2L flasks and was incubated ON at 34°C in dark with shaking. Also before transferring 10 ml cultures, samples were taken and streaked on MPYE+ tet plates and incubated for 2 days at 34°C for appropriate controls to check phenotypes. When the liquid cultures reached to expected value (at $OD_{630} \geq 0.6$), cultures were kept on ice until chromatophore isolation and samples were taken and streaked on MPYE+ tet plates and incubated for 2 days at 34°C for checking phenotypes again.

The cultures kept on ice were centrifuged at 7000 rpm at 4°C for 20 minutes. Supernatants was discarded and *R. capsulatus* cells were resuspended in 40 ml of 50 mM MOPS/100 mM KCl buffer (Appendix B), washed and centrifuged at 8000 rpm at 4°C for 15 minutes. Supernatants was discarded and cells were resuspended in 25 ml ice cold 50 mM MOPS/100 mM KCl buffer and then cells were broken by a double passage through a French pressure cell disrupter (11000 psi). 250 µl PMSF (Phenylmethanosulphonylfluoride) from 100 mM stock was added just before breaking the cells and the sample mixed thoroughly. After breaking the cells, they were homogenated and centrifuged at 12.000 rpm for 45 min at 4°C to remove any unbroken cells. The resulting supernatant was centrifuged at 44.000 rpm for 2 h at 4°C to sediment the membrane fragments. To remove any remaining cytoplasmic proteins the

membrane fragments were washed and resuspended gently in 50 ml of 50 mM MOPS/1 mM KCl buffer (Appendix B) by using paint brush and then centrifuged again at 44.000 rpm for two hours at 4°C. Finally, the pellet was dissolved in 2.5 ml of 50 mM MOPS/100 mM KCl buffer with paint brush than aliquoted and stored at -80°C. Total protein concentration was measured by using Bicinchoninic acid (BCA) method (Stoscheck, 1990). Bovine serum albumin was used as a standard.

2.16.4. SDS-PAGE, Western Blotting, Heme Staining and Coomassie Staining

SDS-PAGE was performed for western blotting on 11% Laemmli (Laemmli, 1970), for heme staining on 16.5% Schagger (Schagger and Von Jagow, 1987) type polyacrylamide gels. Solutions and buffers are given in Appendix B and Appendix C.

For CcoN immunodetection, chromatophore membrane proteins(30mg/ml) on were solubilized in 5% β -mercaptoethanol by incubating at 37°C for 10 minutes before loading on gels. After that, samples were loaded on 11% Laemmli type SDS-PAGE and runned at 90V for 30 minutes than at 120V for 1 hour. After electrophoresis, gels were electroblotted onto Immobilon-P PVDF membranes (Millipore, Billerica, MA). CcoN was detected using anti rabbit polyclonal antibodies raised against *R. capsulatus* CcoN (Koch et al., 1998), and alkaline phosphates-conjugated monoclonal anti-rabbit IgGs Clone RG-16 as a secondary antibody with BCIP/NBT Liquid substrate (Sigma-Aldrich, St. Luise, MO) was used. Before blotting, Immobilon-P PVDF membrane was cut carefully and soaked into methanol (Sigma) for 10 seconds than transferred to Transfer Buffer

(Appendix C) containing petri dish and waited for 10 minutes. Meanwhile six filter papers were soaked into Transfer Buffer to prepare blot sandwich. After electrophoresis, sandwich was prepared by using filter papers, gel and membrane and electroblotted for 1 hour at 0.08V. At the end of 1 hour membrane was carefully removed from the sandwich and put into a petri dish containing 20 ml 5% milk (Appendix B) and incubated ON in cold room with shaking. Next day, membrane was washed three times with 1X TBST Buffer (Appendix C) for 10 minutes. After washing, membrane was incubated with 20 ml 5% milk and 2 μ l CcoN antibody (from 10.000X) for 1 hour with shaking. Then, membrane was washed three times with 1X TBST Buffer for 15 minutes. After washing, membrane was incubated with 20 ml 5% milk and 6 μ l antirabbit IgG alkaline phosphatase conjugate fragment of antibody and incubated for 1 hour with shaking. After incubation with secondary antibody membrane was washed three times with 1X TBST Buffer for 15 minutes. Bands for CcoN appeared after membrane was incubated with BCIP/NBT Liquid substrate for 5 minutes.

The heme peroxidase activity was detected by following the procedure of TMBZ staining (Thomas et al., 1976). For heme staining, 16.5% Schagger type polyacrylamide gels were prepared. First separating, running gel, was prepared and polymerized for 2 hours in cold room then stacking gel was poured in gel apparatus and let it polymerize for another 2 hours in cold room. After polymerization of the gel, chromatophore membrane proteins (50mg/ml) were loaded on SDS-PAGE gel. Before loading, equal volume of the loading dye was added to samples and dissolved in 5% β -mercaptoethanol and incubated at 37°C for 5 minutes. Samples were runned at 30V for 1 hour than at 120V for ON. After

electrophoresis, gel was washed three times with double distilled water. Then, gel was washed with 0.25 M Na-acetate buffer (pH: 5) (Appendix C) for 1 hour. Meanwhile 45 mg 3,3',5,5'-tetramethylbenzidine (TMBZ) was dissolved in 30 ml methanol (6.3 M) and mixed with 70 ml 0.25 M Na- Acetate. Then gel was placed in TMBZ staining solution and washed by shaking gently for 1 hour in dark. Finally, 500µl 30% Hydrogen Peroxide was added and gel was inoculated for 10-30 seconds.

After TMBZ staining, Coomassie staining of the gels were handled. The gels used for heme staining were taken carefully and washed with ddH₂O by shaking gently for 15 minutes. This step was repeated three times. After washing, gels were soaked into Bio-Safe Coomassie G-250 stain (Biorad, Richmond, California) and by gentle shaking incubated for ON. Next day, gels were washed with ddH₂O for several times to get rid of excess amount of stain and visualized.

2.16.5. Cytochrome *c* Oxidase Activity of Chromatophore Membrane Proteins

The cytochrome *c* oxidase activity was carried spectrophotometrically by monitoring the oxidation of reduced horse heart cyt *c* (Sigma, St. Louis, MO) at 550 nm and 25°C using a U3210 UV-visible spectrophotometer (Hitachi) for 2 minutes. Solutions and buffers are given in Appendix B and Appendix C.

Previously prepared chromatophore membrane proteins were solubilized by DDM (Sigma) (final concentration 1%) at 4°C for 2 hours. Unsolubilized cells were removed by centrifugation at 14,000 rpm for 2 hours. Total protein concentration of solubilized proteins was measured by using Bicinchoninic acid

(BCA) method (Stoscheck, 1990). Solubilized proteins were resuspended in assay buffer composed of 10mM Tris-HCl, 120mM KCl (pH:7.0) (Appendix C) to different final concentrations, 1 mg/ml, 3mg/ml and 5 mg/ml. Reduced cytochrome *c* was prepared in assay buffer by adding dithiothreitol (DTT) to a final concentration of 1 mM. The sample was mixed gently and incubated at room temperature for 15 minutes. Afterwards, DTT was removed by gel filtration by using PD-10 (Sigma). To assure the level of reduction is adequate; the absorbance of 20-fold-diluted stock was measured at both 550 and 565 nm. The standard assay mixture (2 ml) was composed of 50 μ l of reduced cyt *c* (final concentration, 4 μ M) and the appropriate volume (1 to 5 μ l) of chromatophore membranes in the assay buffer. Reaction was stopped by adding 100 μ M KCN to the assay mixture. Cytochrome *c* oxidase activity was calculated as micromoles of cytochrome *c* oxidised per mg per minute using an absorption coefficient (ϵ_{550}) of 20.

2.17. Conjugation (Triparental Mating)

To understand the effects of mutations on proton pumping, plasmids pOX15F480A, pOX15 (as positive control) and pRK415 (as negative control) were transferred into KZ101 strain of *R. capsulatus* (unpublished, obtained from Dr. Daldal, University of Pennsylvania, USA) carrying a deletion covering the *NO'* part of the *ccoNOQP* operon [Δ (*ccoNO'*::kan)] and deletion covering the quinol oxidase operon by triparental mating (Ditta et al., 1985) in the presence of pRK2013 as helper cell with the modifications in the procedure. The procedure was followed as described in section 2.14 however after conjugation, plates were incubated for 6 hours at 35°C and then transferred to anaerobic jars and incubated at 35°C under photosynthetic conditions for 3-4 days. After incubation period,

1ml MedA medium was pipeted on the each spot and by using bent glass rod and tipping the plate cells were scraped off the agar surface into the MedA broth and collected at the one edge of the plate and transferred into eppendorf tubes. Then, the cells were centrifuged for 1 minute at 10.000 rpm at 25°C. The supernatant was discarded, and the cells were washed once in 1 ml Med A medium. Washed cells were resuspended in 400 µl Med A medium. 2 µl, 3 µl and 50 µl of the cell suspension were plated on Med A plates with appropriate antibiotics and incubated for 3-4 nights under photosynthetic conditions. At least 4 transconjugants from each cross were purified by picking from the center of well separated colonies and streaked on Med A plate. Transconjugants were selected on the basis of tetracycline and spectinomycin resistance and designated as KZ101/pOX15, KZ101/pRK415 and KZ101/pOX15F480A. The selected transconjugants were stored at -80°C.

2.18. NADI Staining of the KZ101 Strains

After triparental mating, to ensure that each plasmid was transferred successfully to KZ101 strain of *R. capsulatus* and the enzyme is still active quantitative analysis of cyt *cbb*₃ oxidase activity was performed by NADI Staining procedure as described in 2.16.1. Photosynthetically grown KZ101/pOX15, KZ101/pRK415 and KZ101/pOX15F480A strains were tested with NADI reaction. Under these conditions, colonies grown on Med A medium turn deep green within 30 seconds (NADI⁺ phenotype), if they contain an active cyt *cbb*₃ oxidase.

2.19. Whole Cell Proton Pumping Assay

The proton translocation characteristics of the mutants were determined by whole cell pumping assay. The proton translocation was determined by the pH alteration which was monitored by the sensitive pH electrode. Three days before the proton pumping experiments, 1ml MPYE medium supplemented with tetracycline was inoculated with a single colony of KZ101/pOX15, KZ101/pRK415 as positive and negative controls, and mutant strain KZ101/pOX15F480A from a freshly streaked plate and were grown ON (18-24 hours) at 34°C with shaking at 150 rpm. Next day, 1ml culture was transferred to 5 ml MPYE medium supplemented with tetracycline and was grown ON until $OD_{630} \geq 0.6$ at 34°C with shaking at 150 rpm. The day before starting, 5 ml cultures was transferred to 50 ml MPYE medium supplemented with tetracycline in 250 ml flasks and was incubated ON at 34°C with shaking. Also before transferring 10 ml cultures samples were taken and streaked on MPYE+ tet medium and incubated for 2 days at 34°C for appropriate controls to check phenotypes. When the liquid cultures reached to expected value, (at $OD_{630} \geq 0.6$), cells were taken spinned down at 8000 rpm for 20-30 minutes at 4°C. Supernatant was discarded and pellets were resuspended in ice cold freshly prepared 30 ml Hepes Buffer (pH 7.4) (Appendix C) and centrifuged at 6000 rpm for 20 minutes at 4°C. This step was repeated twice. Meanwhile, the proton pumping cell was washed three times with water and ethanol and then with Hepes buffer. pH electrode was calibrated before starting experiments. After centrifugation, pellets were resuspended in 3 ml Hepes buffer and starved approximately for 40 minutes. During this time, the proton pumping cell was filled with 2.8 ml Hepes buffer and

anaerobiosis of the chamber was provided by argon pass, also 1mM HCl was exposed to argon pass too. After 40 minutes starved intact cells (2×10^8) were added to the chamber with 10 μ M myxothiazol and waited for another 20 minutes. Once the pH is stabilized, TMPD was added to the chamber (1mM final concentration) as the electron donor and waited for anaerobiosis for 5 minutes. After anaerobiosis within short period different amounts of oxygen saturated water (5-25 μ l) were pulsed. Same amounts of 1mM HCl were added to compensate the protons consumed and the pH change caused by HCl. HCl was used as the calibration system to calculate the proton pumping efficiency. Up to 100 μ M KCN was added to the solution to testify if the proton translocation was due to cytochrome *c* oxidase.

2.20. Construction of His-tagged Plasmid for the Isolation of *cbb₃* from *R. capsulatus*

2.20.1. Construction of pGY2

For adding a tail of eight histidine codons at the C-terminus of the subunit *ccoP*, pGY2 plasmid was constructed to use as a template for site-directed mutagenesis protocol. A part of *orf277,ccoNOQP* operon of the *cbb₃*-type oxidase and small part of *ccoG*, has already been cloned into pRK415 by using *KpnI* and *XbaI* restriction enzymes by Grzegorz Pawlik, and named as pCcoNHisOQP (unpublished data). This 15.3 kbp plasmid includes six histidine codons immediately upstream of the C-terminus of *ccoN* stop codon; however purification of *cbb₃*-type oxidase from *R. capsulatus* was not able to achieved.

To construct template pGY2 plasmid DNA, pCcoNHisOQP and pBluescript SKII+ DNA were isolated by using GeneJET™ Plasmid Miniprep Kit (Fermentas) and digested with *KpnI* and *SpeI* restriction enzymes (Appendix E) for 1 hour at 37 °C. 10.7 kbp and 4.6 kbp fragments from pCcoNHisOQP plasmid DNA were separated on 1% agarose gel and 4.6 kbp fragment (containing the *ccoNOQP* and small part of *ccoGof cbb₃*-type oxidase from *R. capsulatus*) was isolated from gel by using QIAquick Gel Extraction Kit (Qiagen) (Figure 2.13). Meanwhile *KpnI* and *SpeI* digested pBluescript DNA was separated on 1% agarose gel and ~2.8 kbp fragment was isolated from gel by using QIAquick Gel Extraction Kit (Qiagen) (Figure 2.14). 4.6 kbp fragment was inserted into *KpnI* and *SpeI* site of ~2.8 kbp pBluescript by following the Quick Ligation Kit (NEB) protocol. 5 µl ligation reaction was transformed into One Shot TOP10 chemical competent cells (Invitrogen) and plated on LB plates containing IPTG, X-Gal and ampicillin. Transformants were selected by using Blue/White Colony Assay in the presence of ampicillin (100µg/ml). The clone, 7.4 kbp in size was obtained and designated as TOP10/pGY2 (Figure 2.15).

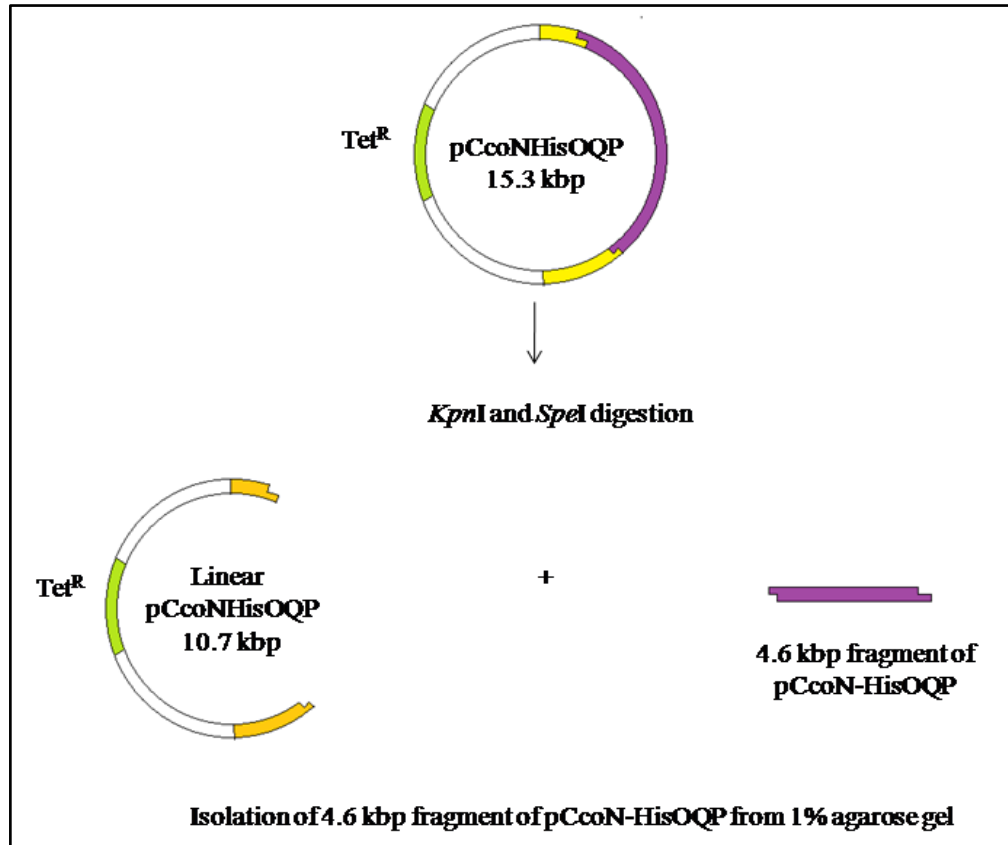


Figure 2.13 Digestion of pCcoNHisOQP DNA with *KpnI* and *SpeI* restriction enzymes

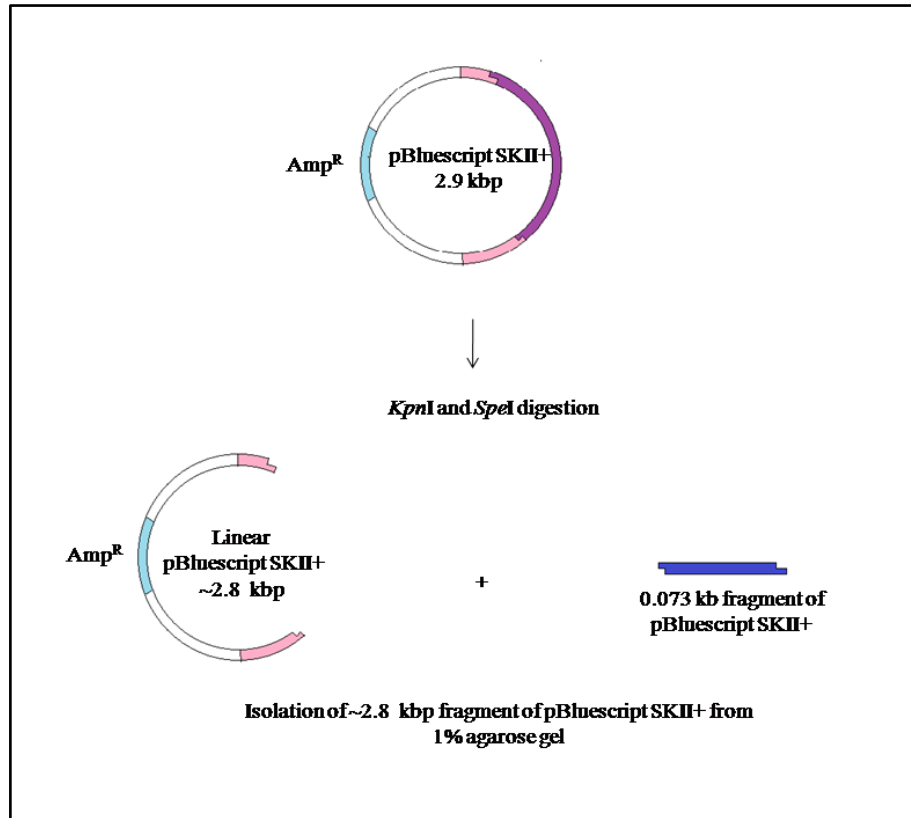


Figure 2.14 Digestion of pBluescript SKII+ DNA with *KpnI* and *SpeI* restriction enzymes

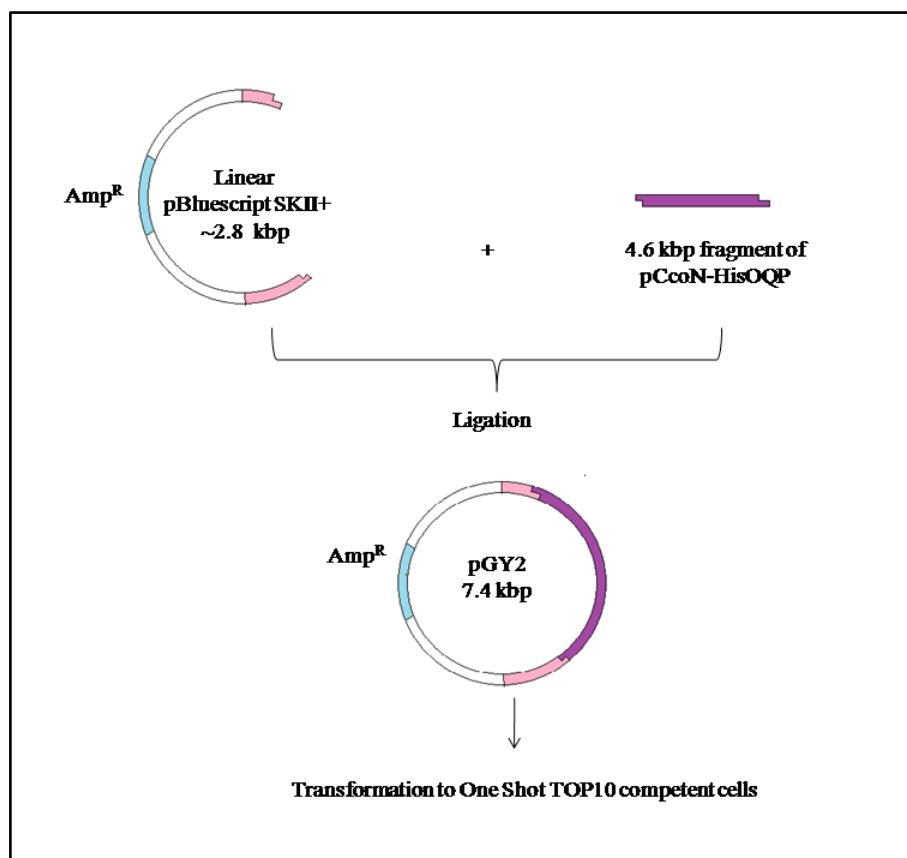


Figure 2.15 Construction of pGY2

2.20.2. Addition of His-Tag to the C- terminus of *ccoP* by PCR Based Site-Directed Mutagenesis

For constructing pGY2CcoNOQP8HIS, eight histidine codons were added to the upstream of the stop codon of *ccoP* by using a two-round Quick Change Site-Directed Mutagenesis (Stratagene) protocol. In the first round, pGY2 was used as a template to add first four histidine codons to the C- terminus of the *ccoP* by using forward and reverse primers, NOQPHisF1 and NOQPHisR1. Forward and reverse primers designed on nucleotide sequence of subunit I of *cbb₃* oxidase in *R. capsulatus* and synthesized by IDT (Table 2.6). PCR reactions were prepared as defined on Table 2.7 and *PfuUltra* polymerase (Stratagene) enzyme was not used in control reaction. All reaction mixtures were incubated at the conditions

shown in Table 2.8. After PCR reactions, PCR products were treated with *DpnI* for 2 hour at 37 °C and 5 µl reaction was transformed into One Shot TOP10 chemical competent cells (Invitrogen) and plated on LB plates containing IPTG, X-Gal and ampicillin. Transformants were selected by using Blue/White Colony Assay in the presence of ampicillin (100µg/ml). Four transconjugants were selected on the basis of colony pigmentation. The plasmid DNA isolation was performed from each transconjugants and sequenced by using primer SeqP (5'-GCCCAACCTCACCGACGGGAT- 3'; 21 kbp) to verify the histidine codons. Sequence verifications were performed at the Biotechnology Center at the University of Illinois, Urbana-Champaign. After sequence verification, the plasmid carrying the first four histidine codons was named as TOP10/pGY2CcoNOQP4HIS. pGY2CcoNOQP4HIS plasmid was used as a template for second step of site-directed mutagenesis with forward and reverse primers, NOQPHisF2 and NOQPHisR2. Same PCR reactions and conditions were used. The plasmid containing eight histidine codons was selected by using Blue/White Colony Assay previously described, and sequence verification was done by using the same SeqP primer. After verification by sequencing it was named as TOP10/pGY2CcoNOQP8HIS.

Table 2.6 Forward and reverse primers used for the site directed mutagenesis of His-tag

Primers	Sequence of the primers
NOQPHisF1	5'TTATGTCCACAGCCTGGGCGGTGGCCAGCATCACCATCACTAACAGC CAAACGGATCCCC 3'
NOQPHisR1	5'GGGGATCCGTTTGGCTGTTAGTGATGGTGATGCTGGCCACCGCCCAG GCTGTGGACATAA 3'
NOQPHisF2	5'CCTGGGCGGTGGCCAGCATCACCATCACCATCACCATCACTAACAG CCAAACGGATCCCC 3'
NOQPHisR2	5'GGGGATCCGTTTGGCTGTTAGTGATGGTGATGGTGATGGTGATGCT GGCCACCGCCCAGG 3'

Table 2.7 PCR reactions for site- directed mutagenesis of His-tag

Reactants	Sample Reaction	Control Reaction
Template DNA (10 ng/μl)	1.5 μl	1.5 μl
Buffer (10X Reactionbuffer-Stratagene)	2.5 μl	2.5 μl
Forward Primer (125 ng/μl)	2 μl	2 μl
Reverse Primer (125 ng/μl)	2 μl	2 μl
dNTP mix (10mM)	1 μl	1 μl
Quickchange solution	1.5 μl	1.5 μl
MgCl ₂ (50mM)	1 μl	1 μl
<i>PfuUltra</i> polymerase (5u/ μl)	1 μl	-
ddH ₂ O	9 μl	10 μl
Total reaction volume	25 μl	25 μl

Table 2.8 PCR conditions for site-directed mutagenesis of His-tag

Cycle	Temperature	Time
First denaturation	95 °C	3 min
Denaturation	95 °C	50 sec
Annealing	58°C	50 sec
Extension	68 °C	7.5 min
Final extension	68 °C	10 min
Number of cycles	18	
Final hold	4 °C	-

2.20.3. Replacement of the 4.6 kbp *NOQP8HIS* Fragment of pGY2CcoNOQP8HIS with that of pCcoNHisOQP

To replace 4.6 kbp *NOQP8HIS* Fragment of pGY2CcoNOQP8HIS with pCcoNHisOQP, plasmid DNAs of pGY2CcoNOQP8HIS and pCcoNHisOQP were isolated by using Wizard[®] Plus SV Minipreps DNA Purification System (Promega). Both of the isolated DNAs were digested *KpnI* and *SpeI* restriction enzymes (Appendix E) for 1 hour at 37°C. After digestion, fragments were separated on the 1% agarose gel and 4.6 kbp fragment from pGY2CcoNOQP8HIS and 10.7 kbp fragment from pCcoNHisOQP were isolated from agarose gel by using QIAquick Gel Extraction Kit (Qiagen) (Figure 2.16). The 4.6 kbp histagged fragment was inserted into 10.7 kbp vector by ligation process at 22°C for 1 hour (Figure 2.17). After ligation process, 5 µl ligation reaction was transformed into One Shot TOP10 chemical competent cells (Invitrogen) and plated on LB plates containing tetracycline. Transformants were selected on the basis of their size (15.3 kbp) and tetracycline resistance. The new clones which have the desired histag were saved and designated as Top10/pCcoNHisOQP8HIS.

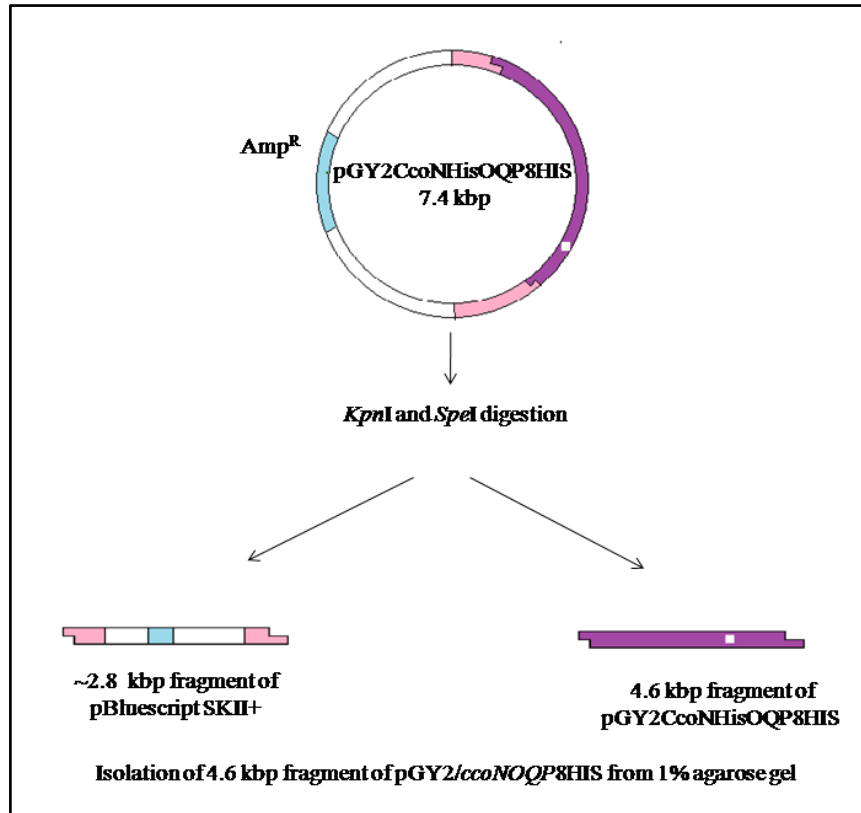


Figure 2.16 Digestion of pGY2CcoNOQP8HIS with *KpnI* and *SpeI* restriction enzymes

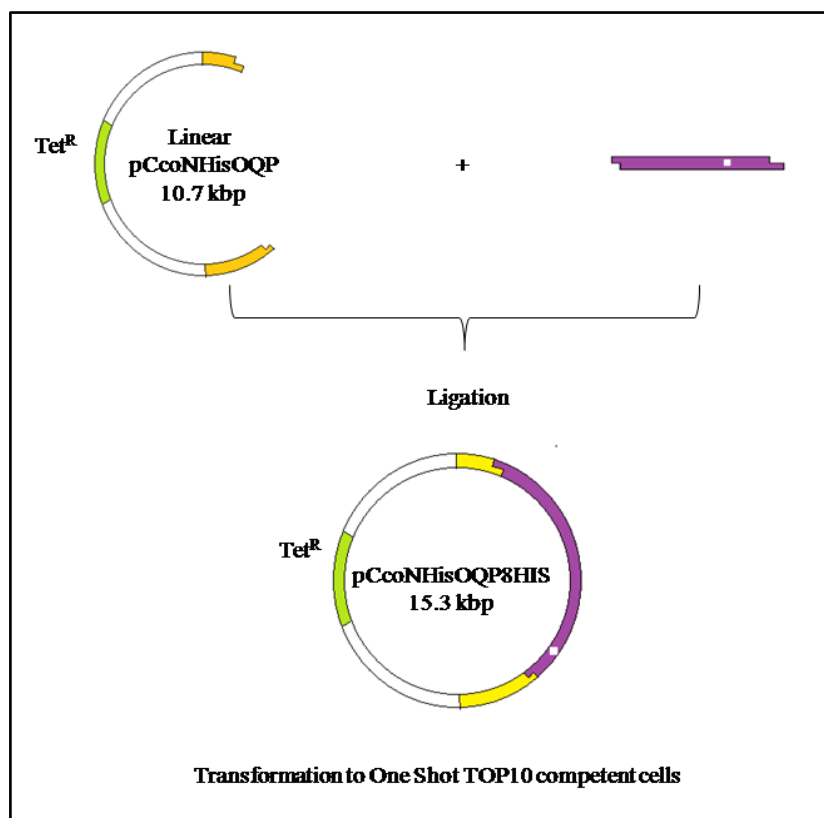


Figure 2.17 Construction of pCcoNHisOQP8HIS

2.20.4. Conjugation

The constructed plasmid pCcoNHisOQP8HIS was transferred into GK32 strain of *R. capsulatus* by triparental mating (Ditta et al., 1985) in the presence of pRK2013 as helper cell. The procedure was followed as described in section 2.14. Transconjugants were selected on the basis of tetracycline and kanamycine resistance and designated as GK32/pCcoNHisOQP8HIS. The selected transconjugants were stored at -80°C.

2.20.5. NADI Staining

After triparental mating, to ensure that plasmid was transferred successfully to GK32 strain of *R. capsulatus* and the enzyme is still active quantitative analysis of cyt *cbb*₃ oxidase activity was performed by NADI Staining procedure as described in 2.16.1. Colonies of GK32/pCcoNHisOQP8HIS were tested with NADI reaction. Under these conditions, colonies grown on Med A medium turn blue within 30 seconds (NADI⁺ phenotype), if they contain an active cyt *cbb*₃ oxidase.

CHAPTER 3

RESULTS

3.1. Construction of pGY as a Template for Y420 and F480 Mutations

The *cbb₃*-type oxidase operon (4.7 kb) has been cloned to pRK404 (10.5 kb) expression vector and named as pOX15 (15.5 kbp) by (Koch et al., 1998). However pRK derivative plasmids are heat sensitive and delicate which may be degraded during PCR reactions, addition to this difficulty, and the size of the pOX15 DNA is too big to be used as a template DNA for site-directed mutagenesis procedure. Thus, usage of pOX15 DNA as a template is not suitable for substituting amino acids for desired ones. pMOZI DNA has been created as a suitable template DNA for mutagenesis of the targeted amino acid (Ozturk et al., 2007). This template DNA is a good candidate for the substitution of Asparagine 265 to Valine 265 however, the positions of the target amino acids and the restriction sites of the enzymes on plasmid, this DNA is not appropriate for Tyrosine 420 and Phenylalaline 480 mutations. To create convenient template DNA, pMOZI DNA was digested with *Xho*I restriction enzyme (Figure 3.1a) and resulting 2.9 kbp and 2.8 kbp fragments were used as vector and insert during ligation process. 10 µl of ligation mixture was transferred into CaCl₂ treated XL1-Blue cells. Transformants were selected on the basis of ampicillin resistance and also by Blue-White colony screening in the presence of IPTG and X-Gal. The clones were tested with *Sph*I and *Sac*I restriction enzymes for the expected orientation. *Sph*I and *Sac*I restriction enzymes cut plasmid

DNA resulting in 5.1 kbp and 0.6 kbp fragments if insert is correctly oriented (pGY) (Figure 3.1b). On the other hand, 3.2 kbp and 2.5 kbp fragments were produced if insert is wrong oriented (pMOZI) (Figure 3.1c). The new clone, 5.7 kbp in size, with correct orientation was designated as XL1-Blue/pGY.

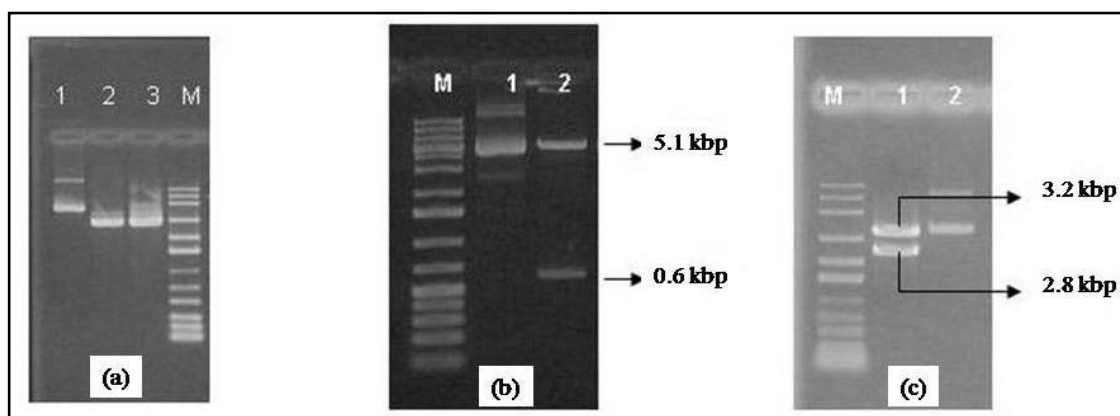


Figure 3.1 Construction of pGY as a Template for Y420 and F480 Mutations. **(a)** Digestion of pMOZI DNA with *XhoI* RE. Line 1 contains uncut pMOZI DNA; Line 2 and Line 3 contain *XhoI* digested pMOZI DNA and separation of 2.9 kbp and 2.8 kbp fragments **(b)** Digestion of pGY DNA with *SphI* and *SacI* RE. Line 1 contains uncut pGY DNA; Line 2 contains *SphI* and *SacI* digested pGY DNA and the separation of 5.1kbp and 0.6 kbp fragments- Correct orientation **(c)** Digestion of pMOZI DNA with *SphI* and *SacI* RE. Line 1 contains *SphI* and *SacI* digested pMOZI DNA and the separation of 3.2 kbp and 2.8 kbp fragments- Wrong orientation; Line 2 contains uncut pMOZI DNA. In each figure Marker is designated as M

3.2. PCR-Based Site- Directed Mutagenesis

The pMOZI DNA was used as a template for the substitution of Asparagine 265 to Valine 265 and pGY DNA was used as template for the substitution of Tyrosine 420 to Phenylalanine 420 and Phenylalanine 480 to Alanine 480 by Quick ChangeTM site-directed mutagenesis protocol using forward and reverse primers. *Pfu* polymerase enzyme which replicates both plasmid strands with high reliability was used. After PCR reactions 10 μ l of PCR products of each mutation and control reaction were visualized on gel (Figure 3.2), each of the PCR products was treated with *DpnI* which digests template DNA and helps to select mutated PCR synthesized

DNA. After *DpnI* treatment, PCR products were loaded agarose gel and electrophorased to see if *DpnI* treatment occurred successfully (Figure 3.3). *DpnI* treated PCR products were transferred into CaCl_2 treated XL1-Blue cells. After incubation period, four single colonies from pGY*Y420, pGY*F480 and pMOZI*N265 were selected from plates and DNAs were isolated and kept at -20°C .

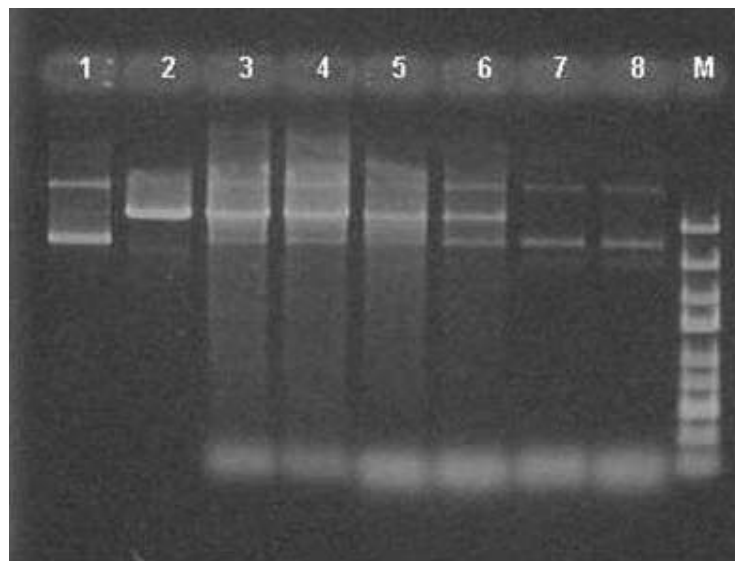


Figure 3.2 PCR products of pGY*Y420, pGY*F480 and pMOZI*N265 (10 μl). Line 1 contains circular pGY DNA as a template; Line 2 contains linear pGY DNA; Line 3 contains pGY*Y420 sample reaction; Line 4 contains pGY*F480 sample reaction; Line 5 contains pMOZI*N265 sample reaction; Line 6 contains pMOZI sample reaction as positive control; Line 7 contains pGY*Y420 control reaction; Line 8 contains pMOZI*N265 control reaction; Marker is designated as M.

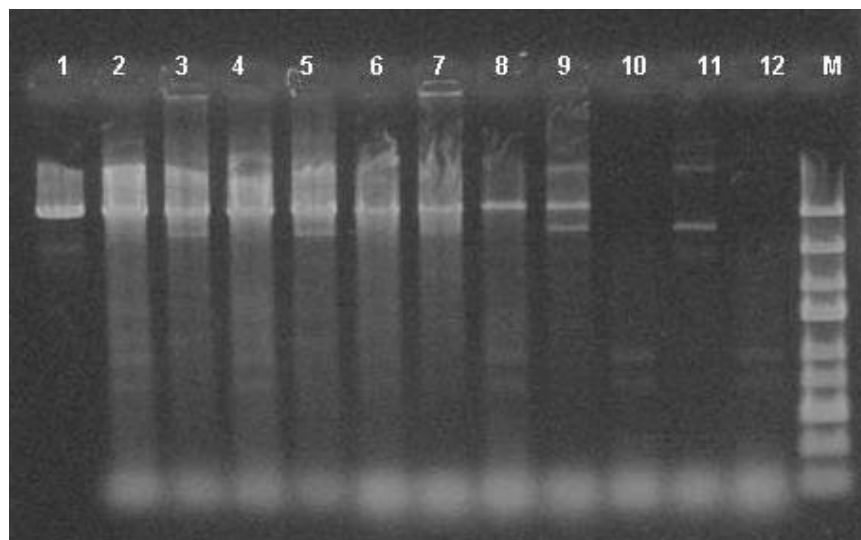


Figure 3.3 *DpnI* treated pGY*Y420, pGY*F480 and pMOZI*N265 PCR(10 μ l) products. Line 1 contains linear pGY DNA; Line 2 contains *DpnI* treated pGY*Y420 sample reaction; Line 3 contains pGY*Y420 sample reaction; Line 4 contains *DpnI* treated pGY*F480 sample reaction; Line 5 contains pGY*F480 sample reaction, Line 6 contains *DpnI* treated pMOZI*N265 sample reaction; Line 7 contains pMOZI*N265 sample reaction; Line 8 contains *DpnI* treated pMOZI sample reaction; Line 9 contains pMOZI sample reaction; Line 10 contains *DpnI* treated pGY*Y420 control reaction; Line 11 contains *DpnI* treated pMOZI*N265 control reaction; Line 12 contains pGY*Y420 control reaction, Marker is designated as M.

3.3. Construction and Characterization of GK32/pOX15N265V Mutant

3.3.1. Sequencing of pMOZIN265V Mutant

Plasmid DNA from pMOZIN265V mutant isolated and sequenced. Obtained sequence was compared with the sequence of the *cbb₃* oxidase from NCBI gene data bank (*Rhodobacter capsulatus* SB 1003 chromosome- gi|294675557:1226693-1228291). It was found that the sequence of pMOZIN265V has desired mutation (GTG) (Figure 3.4). Sequencing with the primers 568F and 214F were provided a sequence for N265 mutation which is long enough to see cloning sites of *EcoRI* and *SphI* restriction enzymes to detect unwanted mutations. Thus there is no other mutation than desired one.

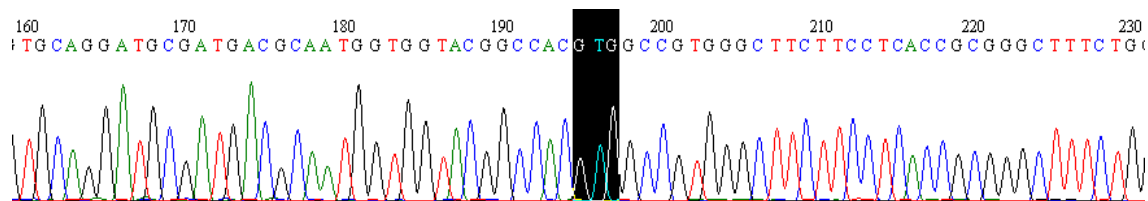


Figure 3.4 The partial sequence of pMOZIN265V transformant. The AAC codon was converted to GTG codon which is labeled.

3.3.2. Replacement of the 0.9 kbp *NO'* Fragment of pMOZII with that of pMOZIN265V Mutant

After creating the desired mutation on the pMOZI DNA which carries the 2.8 kbp fragment of the *cbb₃* oxidase of *R. capsulatus*, next step involves the construction of new mutant containing desired mutation and the whole gene of *cbb₃* oxidase. This step involves the replacement of 2.8 kbp mutated fragment from pMOZI with the pOX15 2.8 kbp fragment. 2.8 kbp fragment was inserted into pBluescript vector by using *Xho*I RE and digestion of pMOZI DNA with *Xho*I RE generates 2.8 kbp and 2.9 kbp fragments which prevents the separation of fragments from each other on agarose gel electrophoresis and obtain the mutated 2.8 kbp fragment. Consequently, obtaining of 2.8 kbp fragment from pMOZI DNA is complicated and required another subcloning. Due to this fact, pMOZII (Oztürk and Mandaci, 2007) DNA is a good candidate for this purpose.

To replace 0.9 kbp fragment of pMOZII with pMOZIN265V, DNAs of pMOZII with pMOZIN265V were digested with *Sph*I and *Eco*RI restriction enzymes. *Sph*I and *Eco*RI digested pMOZII DNA was dephosphorylated by shrimp alkaline phosphatase (SAP) enzyme to prevent self-ligation. The *Sph*I and *Eco*RI digested DNAs of pMOZII and pMOZIN265V were loaded on 1% agarose gel and

electrophoresed for 1 hour. Resulting fragments, 0.9 kbp, 1.6 kbp and 3.2 kbp from pMOZIN265V (Figure 3.5-a) and 5.9 kbp and 0.9 kbp from pMOZII (Figure 3.5-b), were separated successfully. Then 5.9 kbp fragment from pMOZII and 0.9 kbp fragment from pMOZIN265V were isolated from agarose gel (Figure 3.6 and Figure 3.7). The amount of the isolated insert and vector DNA were determined by using agarose gel electrophoresis (Figure 3.8) and 0.9 kbp fragment was inserted into 5.9 kbp fragment from pMOZII and transformed to HB101 competent cells. Transformants were selected on the basis of their size (6.8 kbp) and control digestion with *Sph*I and *Eco*RI RE (Figure 3.9). The new clones which have the desired mutations were saved and designated as HB101/pMOZIIN265V.

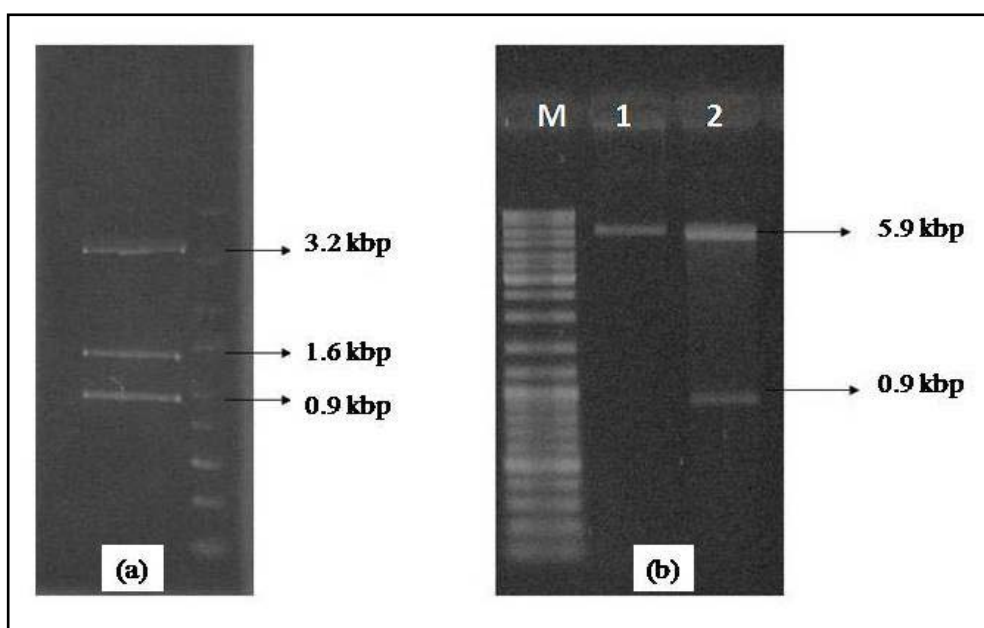


Figure 3.5 Digestion of pMOZIN265V and pMOZII DNA with *Sph*I and *Eco*RI REs. **(a)** Separation of 0.9 kbp, 1.6 kbp and 3.2 kbp fragments from *Sph*I and *Eco*RI digested pMOZIN265V DNA. **(b)** Digestion of pMOZII DNA with *Sph*I and *Eco*RI R. enzymes Line 1 contains uncut pMOZII DNA; Line 2 contains *Sph*I and *Eco*RI digested pMOZII DNA with resulting fragments, 5.9 kbp and 0.9 kbp.

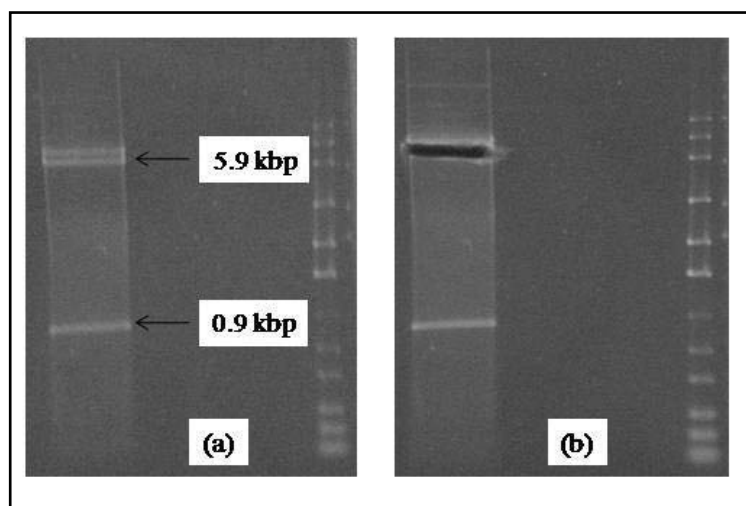


Figure 3.6 Isolation and separation of *Sph*I and *Eco*RI digested pMOZIIDNA. **(a)** Separation of 5.9 kbp and 0.9 kbp fragments from *Sph*I and *Eco*RI digested pMOZII DNA. **(b)** Isolation of 5.9 kbp fragment from 1% agarose gel.

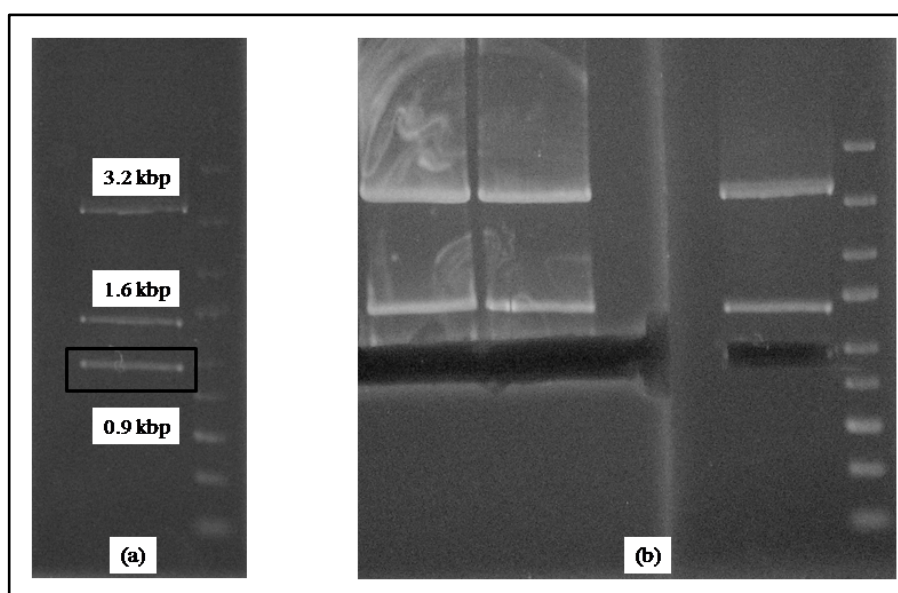


Figure 3.7 Isolation and separation of *Sph*I and *Eco*RI digested pMOZIN265V DNA. **(a)** Separation of 0.9 kbp, 1.6 kbp and 3.2 kbp fragments from *Sph*I and *Eco*RI digested pMOZIN265V DNA. **(b)** Isolation of 0.9 kbp fragment from 1% agarose gel.

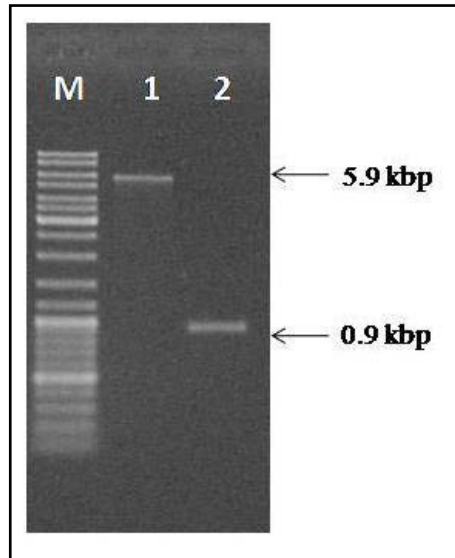


Figure 3.8 Determination of the amount of pMOZII vector DNA and insert DNA. Determination of the amount of 5.9 kbp fragment of *Sph*I and *Eco*RI digested pMOZII vector DNA and 0.9 kbp insert DNA. Line 1 contains the 5.9 kbp fragment of pMOZII vector DNA (100 ng/μl); Line 2 contains 0.9 kbp fragment of insert (45 ng/μl); Marker is designated as M.

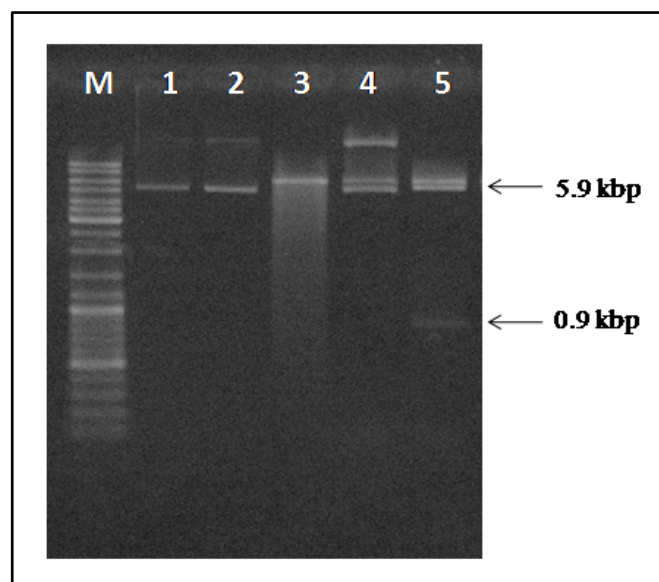


Figure 3.9 Selection of the pMOZIIN265V transformant. Line 1 contains uncut pMOZII DNA as positive control; Line 2 contains possible pMOZIIN265V transformant, Line 3 and Line 4 contain digestion of pMOZIIN265V transformant DNA with *Eco*RI RE; Line 5 contains digestion of pMOZIIN265V transformant DNA with *Sph*I and *Eco*RI REs; Marker is designated as M.

3.3.3. Replacement of the 2.8 kbp NO' Fragment of pOX15 with that of pMOZIIN265V Mutant

To prepare 15.3 kbp pOX15N265V DNA, pMOZIIN265V and pOX12 DNAs were digested with *Xho*I restriction enzyme. *Xho*I digested pOX12 DNA was dephosphorylated by shrimp alkaline phosphatase (SAP) to prevent self-ligation of the vector. Digested samples were loaded on 1% agarose gel and 12.5 kbp fragment from pOX12, 4.0 kbp and 2.8 kbp fragments from pMOZIIN265V were separated. Linear pOX12 DNA (12.5 kbp) and 2.8 kbp fragment from pMOZIIN265V were isolated from agarose gel (Figure 3.10 and Figure 3.11). The amount of vector and insert were further determined by loading of 1 µl DNAs of each on 1% agarose gel before ligation (Figure 3.12). 2.8 kbp fragment from pMOZIIN265V was inserted into linear pOX12 dephosphorylated vector and transformed into DH5α competent cells. Four transformants were selected based on clone size, 15.3 kbp and tetracycline resistance with designation of DH5α /pOX15*N265V.

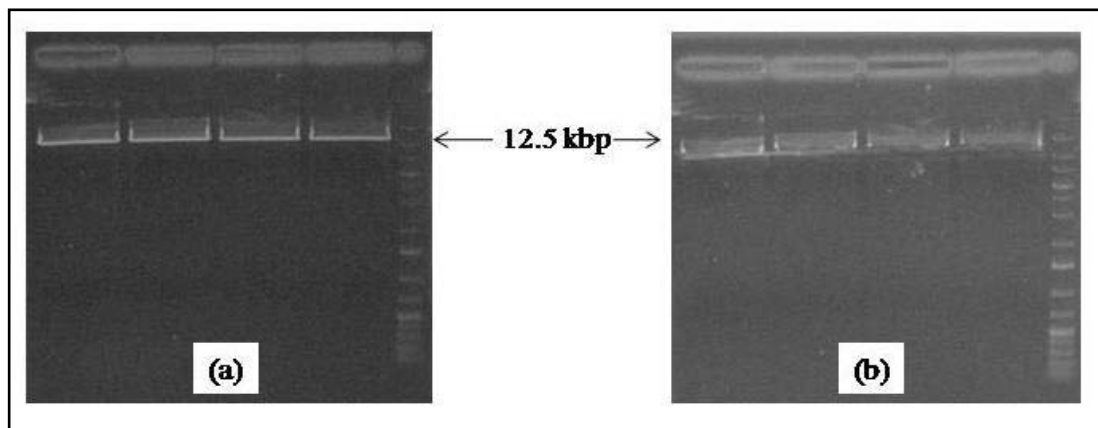


Figure 3.10 Isolation and separation of *Xho*I pOX12 DNA. **(a)** Separation of dephosphorylated 12.5 kbp linear pOX12 DNA. **(b)** Isolation of dephosphorylated 12.5 kbp fragment from 1% agarose gel.

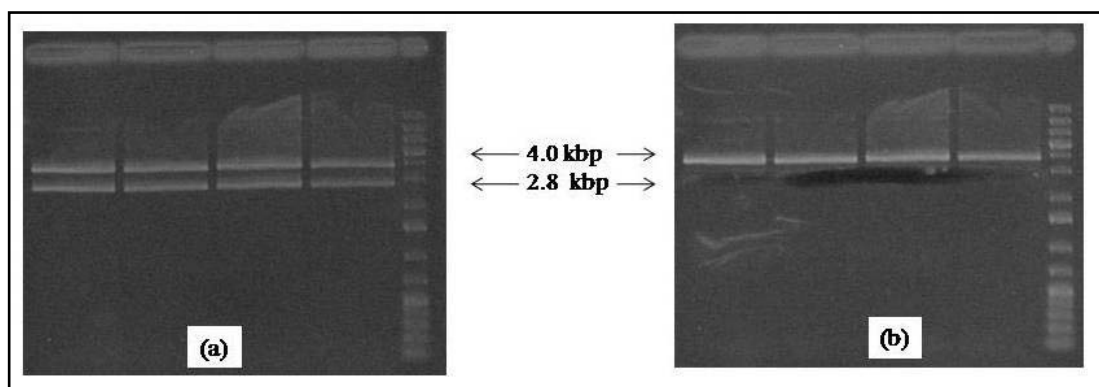


Figure 3.11 Isolation and separation of *XhoI* pMOZIIN265V DNA. **(a)** Separation of *XhoI* digested pMOZIIN265V DNA with resulting fragments, 4.0 kbp and 2.8 kbp. **(b)** Isolation of 2.8 kbp fragment from 1% agarose gel.

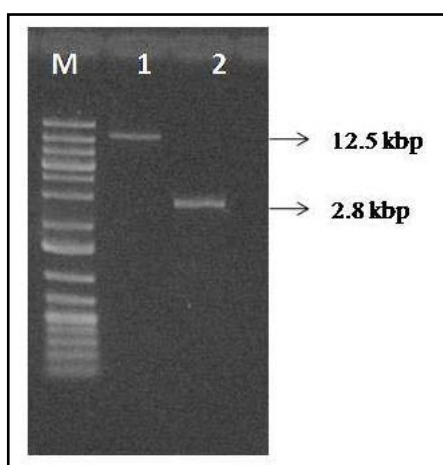


Figure 3.12 Determination of the amount dephosphorylated pOX12 vector DNA and insert DNA from pMOZIN265V. Determination of the amount of 12.5 kbp fragment of *XhoI* digested and dephosphorylated pOX12 vector DNA and 2.8 kbp insert DNA from pMOZIN265V. Line 1 contains the 12.5 kbp fragment of dephosphorylated pOX12 vector DNA (100 ng/μl); Line 2 contains 2.8 kbp fragment of insert (150 ng/μl) from pMOZIIN265V; Marker is designated as M.

3.3.4. Control of the 2.8 kbp Insert Orientation of pOX15*N265V Mutant

To select the clone that has correct oriented insert, four different plasmids DNAs from pOX15*N265V transformants were isolated and digested with *Hind*III RE. *Hind*III cuts vector and insert once, resulting 3.6 kbp and 11.7 kbp fragments if the insert is correctly oriented, but 2.8 kbp and 12.5 kbp fragments appear if the insert is wrong oriented. After digestion of pOX15*N265V DNAs from transformants, it was found that two of them are correctly oriented (Figure 3.13; Lines 5 and 6) and the others were wrong oriented (Figure 3.13; Lines 3 and 4). The new clone that have correct oriented mutated insert were kept at -80°C and named as DH5 α /pOX15N265V.

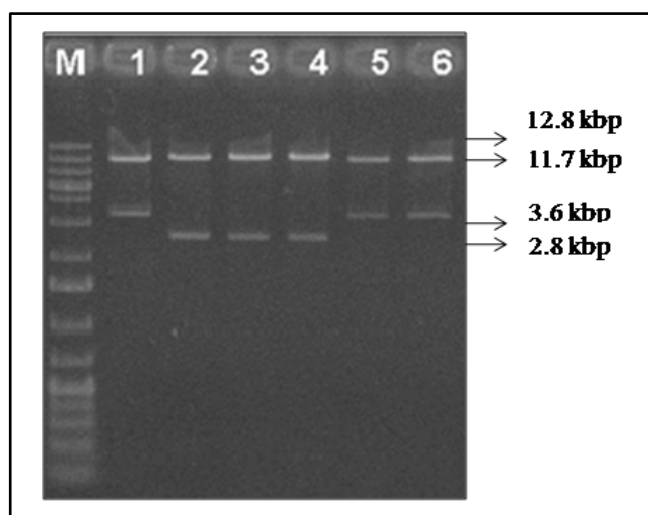


Figure 3.13 *Hind*III Digestion of DH5 α /pOX15*N265V transformants. Line 1 contains control of correct orientation-3.6 kbp and 11.7 kbp fragments; Line 2 contains control of wrong orientation-2.8 kbp and 12.5 kbp fragments; Line 3 and 4 contain *Hind*III digested pOX15*N265V DNA-wrong oriented; Line 5 and 6 contain *Hind*III digested pOX15*N265V DNA-correct oriented; Marker is designated as M.

3.3.5. Transformation of pOX15N265V to *R. capsulatus* by Triparental Mating

The pOX15N265V plasmid DNA that is in DH5 α strains of *E. coli* was transferred into GK32 strain of *R. capsulatus* in the presence of HB101/pRK2013 helper cells. Four different transconjugants were selected based on tetracycline, kanamycin resistance. Selected transconjugants were named as GK32/pOX15N265V and kept at -80°C for further analysis.

3.3.6. Determination of *cbb*₃ Oxidase Phenotype Profile of GK32/pOX15N265V Mutant with NADI Staining

The effect of amino acid replacements on cytochrome *cbb*₃oxidase activity was visually determined by using the NADI Staining test which allows rapid screening for cytochrome *c* dependent oxidase activity by observation of colony pigmentation. NADI staining was performed with whole cells of GK32/pOX15, as positive control, GK32, as negative control, and targeted mutant GK32/pOX15N265V that had been grown under aerobic conditions. Colonies that exhibit the cytochrome *c* oxidase activity turn blue (indophenols blue) by the treatment with a 1:1 (vol/vol) mixture of 35 mM α -naphthol in ethanol and 30 mM *N,N*-deimethy-*p*-phenylenediamine in water. Under these conditions colonies with deep blue coloration after 30 seconds designated as NADI positive (NADI⁺ phenotype).

After NADI staining, the positive control strain, GK32/pOX15 showed *cbb*₃ oxidase activity (Figure 3.14a), on the other hand, the negative control strain GK32 showed virtually no *cbb*₃ oxidase activity as expected(Figure 3.14b). The mutant GK32/pOX15N265V was also showed virtually no *cbb*₃ oxidase activity (Figure 3.14c) similar to negative control strain, GK32. NADI staining phenotype of

GK32/pOX15N265V mutant indicates that the Asparagine 265 is a critical amino acid for enzyme activity or assembly.

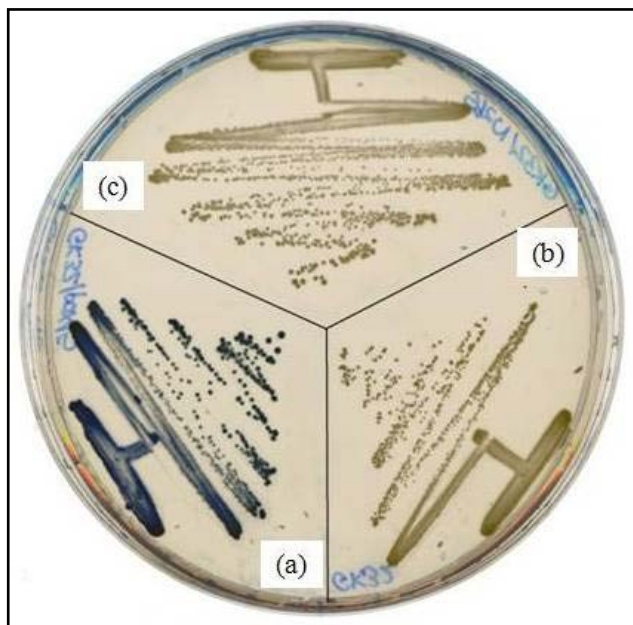


Figure 3.14 NADI stained plate of GK32/pOX15N265V mutant. **(a)** represents NADI stained GK32/pOX15 strain as positive control with NAD⁺ phenotype; **(b)** represents NADI stained *R. capsulatus* GK32 strain as negative control with NAD⁻ phenotype; **(c)** represents NADI stained GK32/pOX15N265V mutant with NAD⁻ phenotype.

3.3.7. Re-sequencing of Mutant GK32/pOX15N265V

After substitution of target amino acids to desired ones by PCR-based site-directed mutagenesis technique, plasmid DNAs of each mutant was sequenced for the verification of the mutations. The sequence results revealed all mutations were created successfully and no other mutations were detected. Before starting the characterization experiments, plasmid DNA from GK32/pOX15N265V mutant strain was isolated and sequenced at DNA Sequencing Facility of University of Pennsylvania to make sure that plasmid carries the desired mutation. Sequence

obtained from plasmid DNA was compared with the sequence of the *cbb₃* oxidase from NCBI Gene Databank and found that sequence has desired mutation and there is no mutation other than desired one (Figure 3.15).

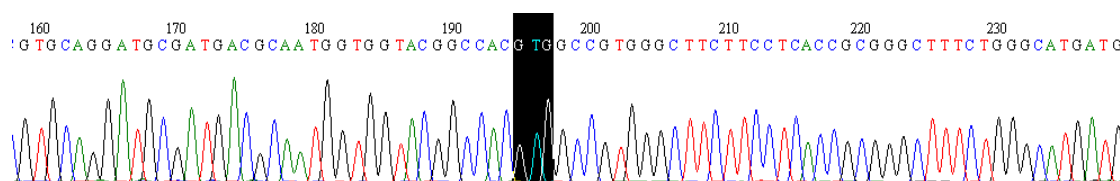


Figure 3.15 The partial sequence of GK32/pOX15N265V transformant. The AAC codon was converted to GTG codon.

3.3.8. Detection of CcoN in GK32/pOX15N265V Mutant by Western Blot Analysis

The presence of subunit I (CcoN) of the cyt *cbb₃* oxidase were tested with polyclonal antibodies raised against purified CcoN obtained from *R. capsulatus* MT1131, as described in (Koch et al., 1998). For CcoN immunodetection, chromatophore membrane proteins from MT1131 (wild type), GK32 (negative control mutant), GK32/pOX15 (positive control mutant) GK32/pOX15N265V were electroblotted onto Immobilon-P membranes and immunoglobulins bound to crossreacting *R. capsulatus* proteins were detected with alkaline phosphates-conjugated monoclonal anti-rabbit secondary antibodies. Western blot analyses revealed that CcoN was detected in MT1131 (wild type) (Figure 3.16, Line 4) and in GK32/pOX15 (positive control mutant) (Figure 3.16, Line 2) however, CcoN was absent in GK32 (negative control mutant) (Figure 3.16, Line 3) as expected. The western blot analysis of GK32/pOX15N265V mutant showed that this mutant lacked CcoN of the cyt *cbb₃* oxidase (Figure 3.16, Line 1).

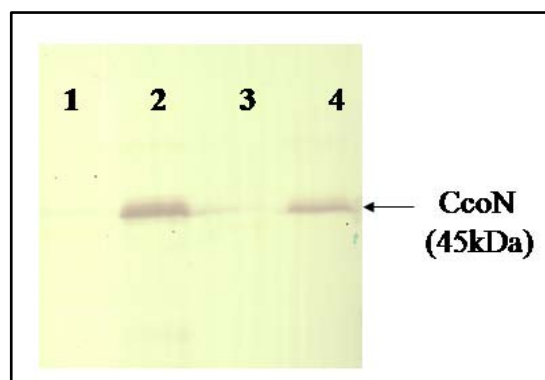


Figure 3.16 Western blot analyses of GK32/pOX15N265V mutant. Western blot analyses with anti-CcoN (subunit I) antibodies. After SDS-PAGE (10 µg of membrane proteins per lane) electrophoretically transferred onto an Immobilon-P membrane. Line 1 contains GK32/pOX15N265V; Line 2 contains GK32/pOX15, as positive complemented mutant; Line 3 contains GK32, as negative control; Line 4 contains wild-type, MT1131.

3.3.9. Cytochrome Profiles of GK32/pOX15N265V Mutant

By using the SDS/PAGE-TMBZ staining technique, membrane-bound *c*-type cyts with molecular masses of 32, 31, 29 and 28 kDa of *R. capsulatus* can be visualized by their peroxidase activity. The 32 kDa protein is the cyt c_1 subunit of the cyt bc_1 complex (Jenney and Daldal, 1993; Gray et al., 1994; Jenney et al., 1994), and the 29-kDa protein is the membrane-associated electron carrier cyt c_y (Jenney and Daldal, 1993). The two remaining cyts of 32 and 28 kDa (cyts c_p and c_o , respectively) correspond to the heme *c*-containing subunits of the cyt cbb_3 oxidase.

For the detection of four distinct membrane-bound *c*-type cyts, desired mutant GK32/pOX15N265V, MT1131, GK32/pOX15 and GK32 were analyzed. TMBZ staining results revealed that mutant GK32/pOX15N265V has no CcoP and CcoO subunits (Figure 3.17, Line 1), which is similar to negative control strain, GK32 (Figure 3.17, Line 3).

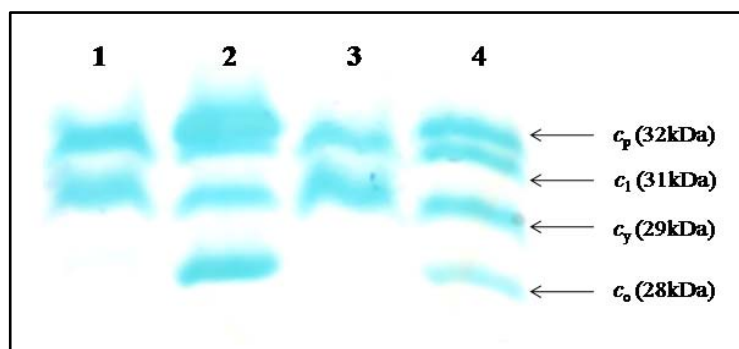


Figure 3.17 Cytochrome *c* profile of GK32/pOX15N265V mutant together with wild type, MT1131, positive control strain, GK32/pOX15, and negative control strain, GK32. Cytochrome *c* profiles of chromatophore membranes of *R.capsulatus* strains grown in enriched MPYE medium under respiratory conditions. Approximately 50 μ g of protein was loaded in each line of a 16.5% SDS polyacrylamide gel. After electrophoresis, *c*-type cyts were detected by TMBZ staining. Line 1 contains GK32/pOX15N265V; Line 2 contains GK32/pOX15, as positive complemented mutant; Line 3 contains GK32, as negative control; Line 4 contains wild-type, MT1131. cyt *cp* and *co* are the subunits II and III of cyt *cbb*₃ oxidase, and cyts *c*₁ and *cy* correspond to the cyt *c*₁ subunit of the *bc*₁ complex and the membrane-attached electron carrier *cy*, respectively.

On the other hand, Coomassie Brilliant Blue staining of the gel was also supported TMBZ staining results (Figure 3.18). Together with western blotting results, it had been showed that GK32/pOX15N265V mutant lacks all subunits of the cyt *cbb*₃ oxidase. Thus, it could be said that Asparagine at position of 265 is important for cyt *cbb*₃-type oxidase assembly.

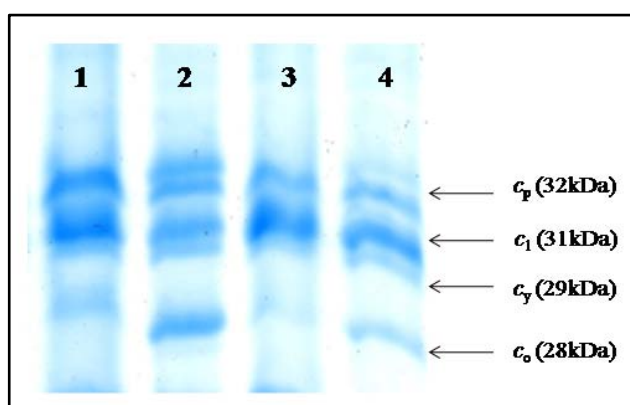


Figure 3.18 Coomassie Brilliant Blue staining of GK32/pOX15N265V mutant. Line 1 contains GK32/pOX15N265V; Line 2 contains GK32/pOX15, as positive complemented mutant; Line 3 contains GK32, as negative control; Line 4 contains wild-type, MT1131. cyt *cp* and *co* are the subunits II and III of cyt *cbb*₃ oxidase, and cyts *c*₁ and *cy* correspond to the cyt *c*₁ subunit of the *bc*₁ complex and the membrane-attached electron carrier *cy*, respectively.

3.3.10. Cytochrome *c* Oxidase Activity of GK32/pOX15N265V Mutant

The cytochrome *c* oxidase activity of chromatophore membranes MT1131, GK32/pOX15, GK32 and mutant GK32/pOX15N265V were measured spectrophotometrically by monitoring the oxidation of reduced horse heart cyt *c*. It had been shown that GK32/pOX15 membranes have 2.5 to 3 times higher *cbb*₃ - cytochrome *c* oxidase activity than in wild-type membranes, as a result of considering a copy number of three to five copies/cell for pRK415 derived plasmids (Peters et al., 2008). According to cytochrome *c* oxidase activity measurements, the complement mutant GK32/pOX15 has approximately 2 times higher activity than the wild type control, MT1131. As the mutations are on the pOX15 plasmid, *cbb*₃ oxidase activity of the complement mutant was accepted as 100% and *cbb*₃ oxidase activity of the wild type and mutants were calculated compared to this value. For cytochrome *c* oxidation, 100% activity corresponds to 1602 nmol of cyt *c*/mg of protein per minute. The values for the *cbb*₃ oxidase activity were calculated as the means of three independent measurements.

The measurements of *cbb*₃ oxidase activity results indicated that the wild type control, MT1131 had 47% activity and negative control had no activity for *cbb*₃ oxidase as expected. The GK32/pOX15N265V mutant revealed 1% activity of *cbb*₃ oxidase, which conforms its negative cyt *c* oxidase phenotype resulted from NADI Staining.

All data support that Asparagine residue at position of 265 is important for the structural characteristic of cyt *cbb*₃-type oxidase from *R. capsulatus*.

3.4. Construction and Characterization of GK32/pOX15Y420F Mutant

3.4.1. Sequencing of pGY*Y420F Mutant

Plasmid DNA from pGY*Y420F mutant was isolated and sequenced. Sequences obtained from plasmid DNA was compared with the sequence of the *cbb₃* oxidase from NCBI gene data bank. Sequence results showed that, pGY*Y420F sequence has the desired TTC (reverse complement: AAG) mutation (Figure 3.19). For Y420 mutation, universal M13R-PUC primer was used, this primer was sequenced the plasmids reverse thus sequence results would be reverse complement of the original sequence of the *cbb₃* oxidase. Sequencing results showed that there is no other mutation than desired one created between restriction enzymes sites.

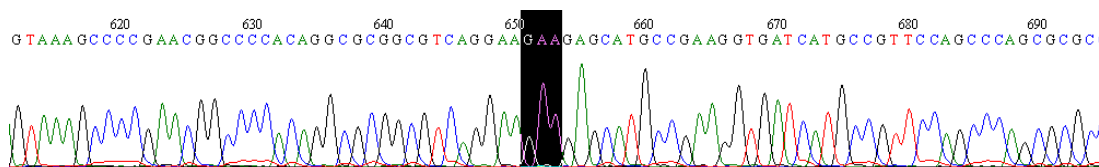


Figure 3.19 The partial sequence of pGY*Y420F transformant. The TAC codon was converted to TTC codon which is labeled (as reverse complement GAA).

3.4.2. Replacement of the 0.6 kbp NO' Fragment of pGY with that of pGY*Y420F Mutant

For the replacement of 2.8 kbp mutated fragment from pGY mutants with the pOX15 2.8 kbp fragment, a subcloning was needed. Even the 0.6 kbp mutated fragment of each transformant was sequenced and no other unwanted mutations were detected in the sequenced part, there might be still unwanted mutations at the rest of

the plasmid (5.1 kbp). Sequencing of 5.1 kbp fragment for each mutant is time consuming and expensive. Due to this reason, another subcloning was performed for Y420 and F480 mutations.

To replace 0.6 kbp fragment of pGY with that of pGY*Y420F, DNAs from pGY and pGY*Y420F were isolated and digested with *Sph*I and *Sac*I restriction enzymes. *Sph*I and *Sac*I digested pGY DNA was dephosphorylated by shrimp alkaline phosphatase (SAP) to prevent self-ligation of vectors with each other. The *Sph*I and *Sac*I digested pGY and pGY*Y420F DNAs were loaded on 1% agarose gel and resulting fragments, 0.6 kbp, and 5.1 kbp, were separated (Figure 3.20). After separation, 5.1 kbp fragment from pGY, and 0.6 kbp fragment from pGY*Y420F were isolated from agarose gel (Figure 3.21 and Figure 3.22). The amounts of the isolated insert and vector were determined by using gel electrophoresis (Figure 3.23). Finally 0.6 kbp fragment from pGY*Y420F was inserted into 5.1 kbp fragment from pGY and transformed to XL1-Blue competent cells. Four different transformants were selected on the basis of their size (5.7 kbp) and their ampicillin resistance. Plasmid DNAs from four different plasmids were isolated and sequenced to ensure that each transformant has the desired mutation. After verifying the mutations new clones were saved and designated as XL1-Blue/pGYY420F.

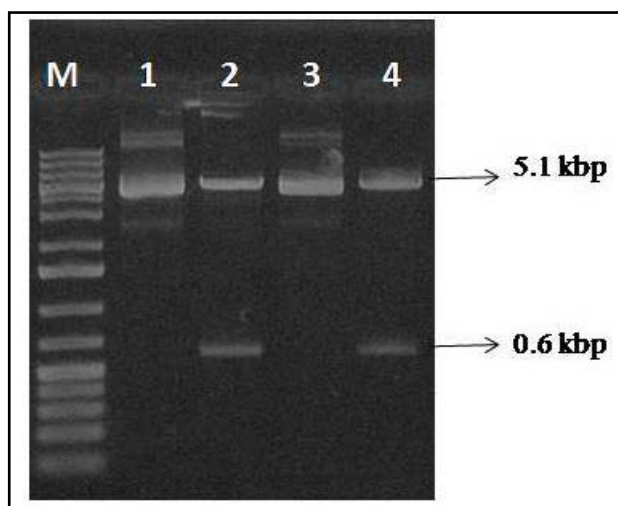


Figure 3.20 Digestion of pGY and pGY*Y420F DNAs with *SphI* and *SacI* Res. Line 1 contains uncut pGY DNA as control; Line 2 contains *SphI* and *SacI* digested dephosphorylated pGY DNA with resulting fragments, 5.1 kbp and 0.6 kbp; Line 3 contains uncut pGY*Y420F DNA as control; Line 4 contains *SphI* and *SacI* digested pGY*Y420F DNA with resulting fragments, 5.1 kbp and 0.6 kbp. Marker is designated as M.

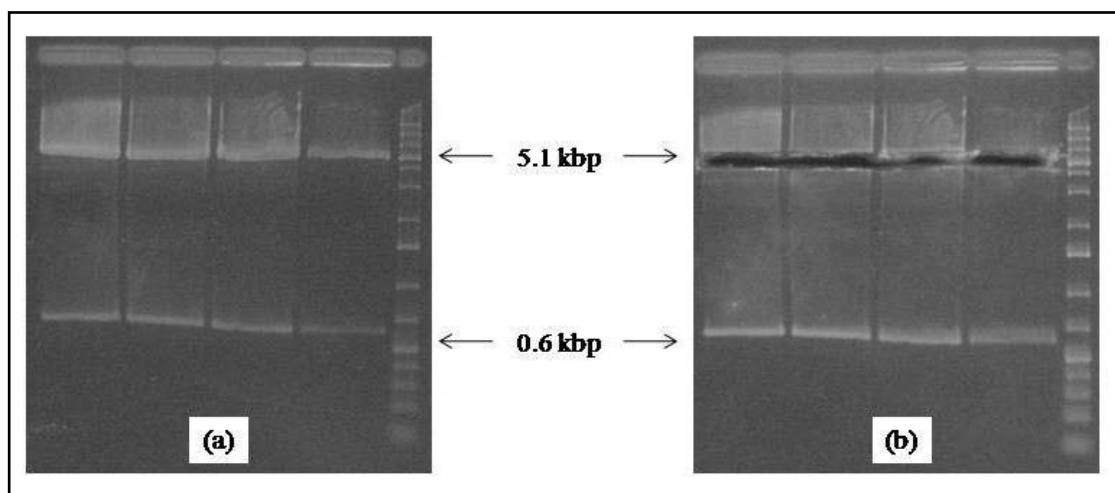


Figure 3.21 Isolation and separation of *SphI* and *SacI* digested dephosphorylated pGY DNA. **(a)** Separation of 5.1 kbp and 0.6 kbp fragments from *SphI* and *SacI* digested pGY DNA. **(b)** Isolation of 5.1 kbp fragment from 1% agarose gel

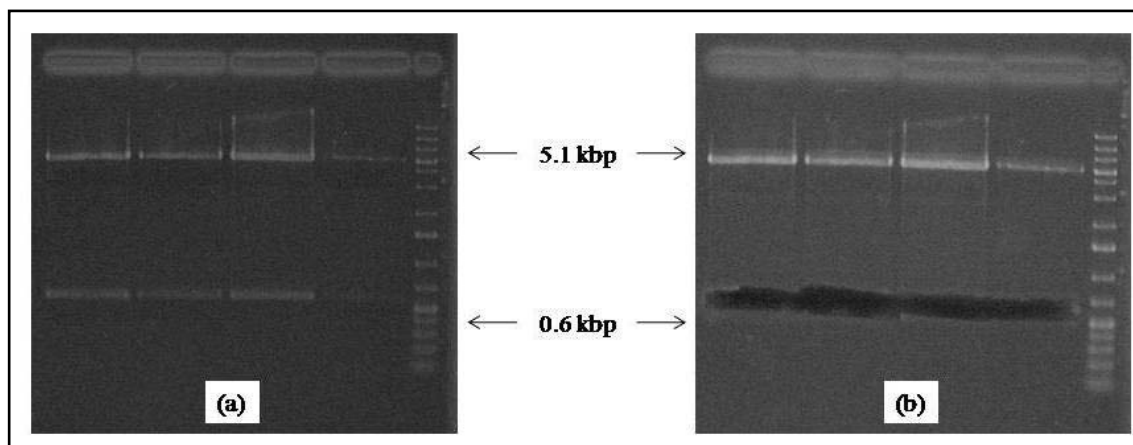


Figure 3.22 Isolation and separation of *SphI* and *SacI* digested pGY*Y420F DNA. **(a)** Separation of 5.1 kbp and 0.6 kbp fragments from *SphI* and *SacI* digested pGY*Y420F DNA. **(b)** Isolation of 0.6 kbp fragment from 1% agarose gel.

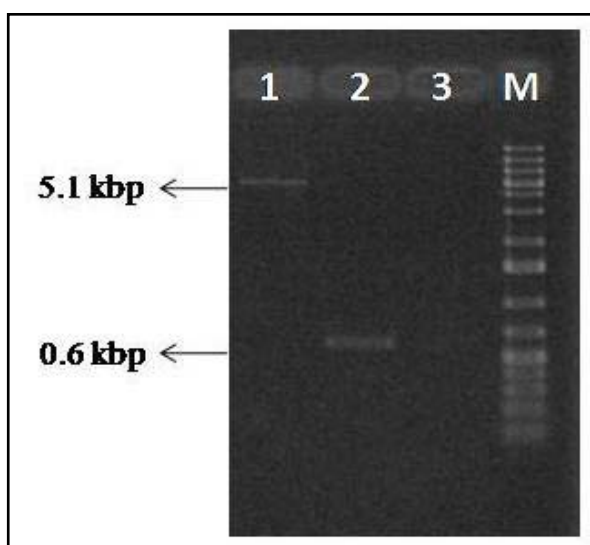


Figure 3.23 Determination of the amount of dephosphorylated pGY vector DNAs. Determination of the amount of 5.1 kbp fragment of *SphI* and *SacI* digested dephosphorylated pGY vector DNA and 0.6 kbp insert DNA from pGY*. Line 1 contains the 5.1 kbp fragment of pGY vector DNA (10 ng/μl); Line 2 and 3 contain 0.6 kbp fragment of insert (15 ng/μl), (5 ng/μl) from pGY*Y420F. Marker is designated as M.

3.4.3. Replacement of the 2.8 kbp NO' Fragment of pOX15 with that of pGYY420F Mutant

For the preparation of 15.3 kbp pOX15Y420F DNA, pGYY420F DNA was digested by *Alw441* and *XhoI* restriction enzymes. *Alw441* restriction enzyme digests pBluescript DNA twice thus, 2.8 kbp mutated fragment can be isolated easily. pOX12 DNA was digested with *XhoI* restriction enzyme. *XhoI* digested pOX12 DNA was dephosphorylated by shrimp alkaline phosphatase (SAP) to prevent self-ligation of the vector. Digested samples were loaded on 1% agarose gel and 12.5 kbp fragment from pOX12, and 2.8 kbp, 1.3 kbp, 0.9 kbp and 0.7 kbp fragments from pGYY420F were separated (Figure 3.24). Linear pOX12 DNA and 2.8 kbp fragments from pGYY420F were isolated from agarose gel (Figure 3.25 and Figure 3.26). Also, the amounts of vector and insert were determined by loading of 1 μ l DNAs of each on 1% agarose gel before ligation (Figure 3.27). Finally, 2.8 kbp fragment from pGYY420F was inserted into dephosphorylated linear pOX12 vector and transformed into DH5 α competent cells. Four different transformants were selected based on clone size, 15.3 kbp and tetracycline resistance. The new clones were designated as DH5 α /pOX15*Y420F before orientation check of the insert.

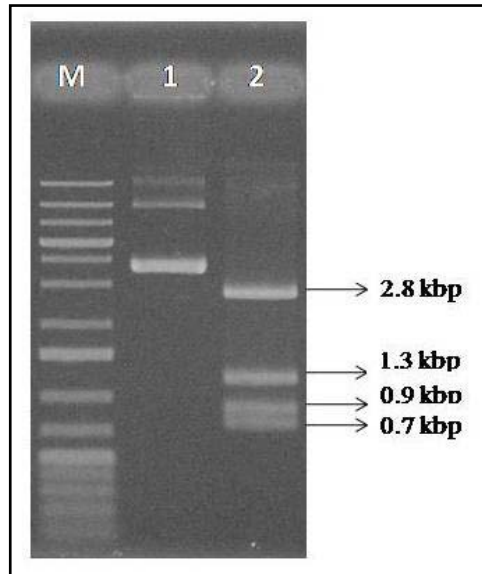


Figure 3.24 Separation of *Alw*441 and *Xho*I digested pGYY420F DNA. Line 1 contains uncut pGYY420F DNA; Line 2 contains *Alw*441 and *Xho*I digested pGYY420F DNA with resulting fragments, 2.8 kbp, 1.3 kbp, 0.9 kbp and 0.7 kbp; Marker is designated as M.

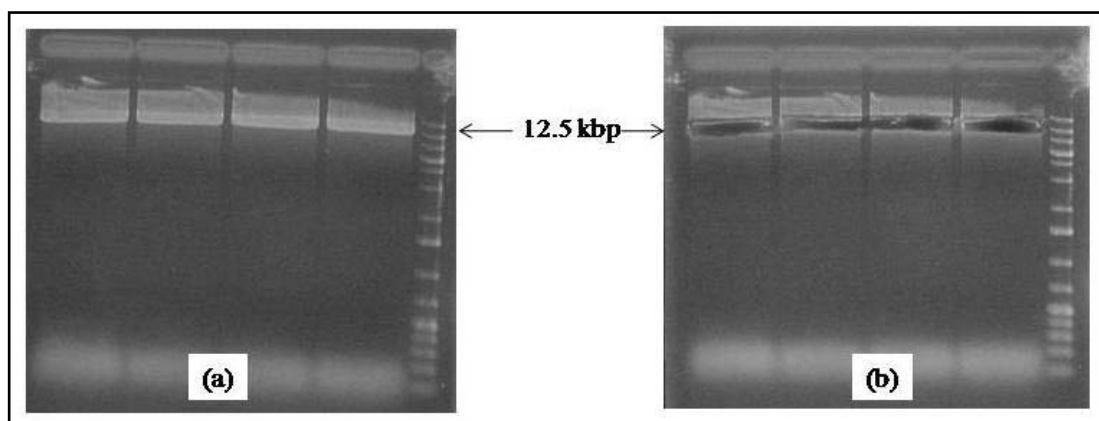


Figure 3.25 Isolation and separation of *XhoI* pOX12 DNA. **(a)** Separation of dephosphorylated 12.5 kbp linear pOX12 DNA. **(b)** Isolation of dephosphorylated 12.5 kbp fragment from 1% agarose gel.

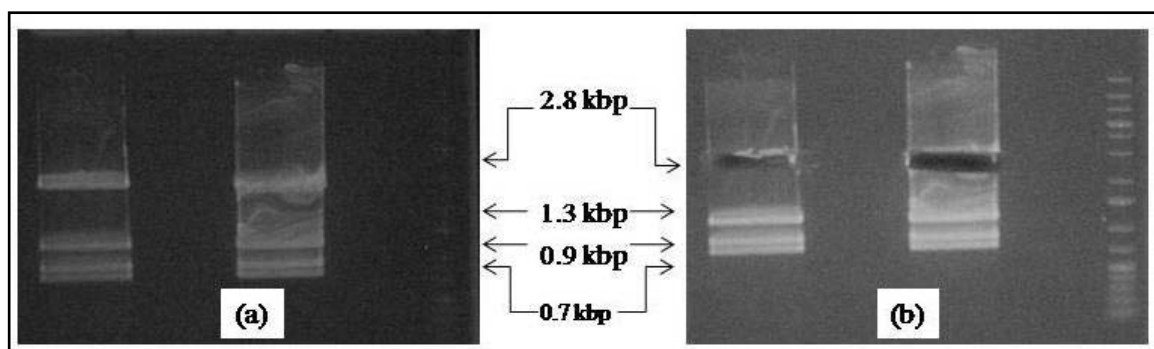


Figure 3.26 Isolation and separation of *Alw441* and *XhoI* digested pGYY420F DNA. **(a)** Separation of *Alw441* and *XhoI* digested pGYY420F DNA with resulting fragments, 2.8 kbp, 1.3 kbp, 0.9 kbp and 0.7 kbp. **(b)** Isolation of 2.8 kbp fragment from 1% agarose gel.

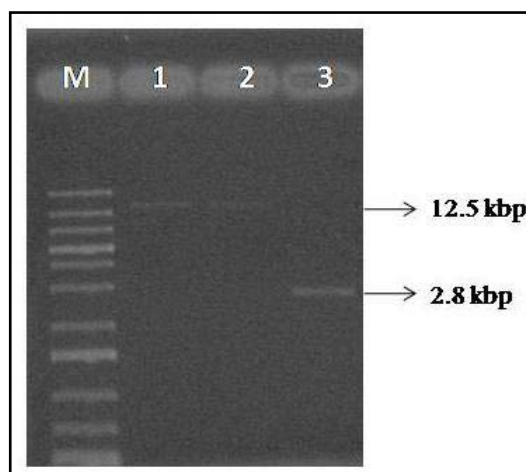


Figure 3.27 Determination of the amount of dephosphorylated pOX12 vector DNA. Determination of the amount of 12.5 kbp fragment of *Xho*I digested and dephosphorylated pOX12 vector DNA and 2.8 kbp insert DNA pGYY420F DNA. Line 1 and Line 2 contain the 12.5 kbp fragment of dephosphorylated pOX12 vector DNA (10 ng/ μ l) and (5 ng/ μ l) respectively; Line contains 2.8 kbp fragment of insert (30 ng/ μ l) from pGYY420F; Marker is designated as M.

3.4.4. Control of the 2.8 kbp Insert Orientation of pOX15*Y420F Mutant

To select the clone that has correct oriented insert, two plasmids of pOX15*Y420F were isolated from two different transformants and digested with *Hind*III RE. *Hind*III cuts vector and insert once, resulting 3.6 kbp and 11.7 kbp fragments if the insert is correctly oriented, but 2.8 kbp and 12.5 kbp fragments appear if the insert is wrong oriented. The digestion of pOX15*Y420F transformant DNAs by *Hind*III restriction enzyme resulted that one of them is correctly oriented (Figure 3.28, Line 4) and the other one is wrong oriented (Figure 3.28, Line 3). The new clone that have correct oriented mutated insert were kept at -80°C and named as DH5 α /pOX15Y420F.

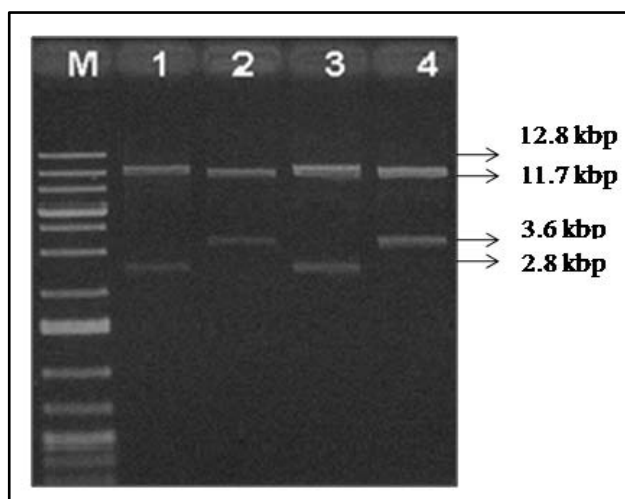


Figure 3.28 *Hind*III Digestion DH5 α /pOX15*Y420F transformants. Line 1 contains control of wrong orientation-2.8 kbp and 12.5 kbp fragments; Line 2 contains control of correct orientation-3.6 kbp and 11.7 kbp fragments; Line 3 contains *Hind*III digested pOX15*Y420F DNA-wrong oriented; Line 4 contains *Hind*III digested pOX15*Y420F DNA-correct oriented; Marker is designated as M.

3.4.5. Transformation of pOX15Y420F to *R. capsulatus* Using Triparental Mating

The pOX15Y420F plasmid DNA that is in DH5 α strains of *E. coli* was transferred into GK32 strain of *R. capsulatus* in the presence of HB101/pRK2013 helper cells. Transconjugants were selected based on tetracycline, kanamycin resistance and named as GK32/pOX15Y420F were selected and kept for further analysis at -80°C.

3.4.6. Determination of *cbb*₃ Oxidase Phenotype Profile of GK32/pOX15Y420F Mutant with NADI Staining

After NADI staining of the complemented control mutant, GK32/pOX15, negative control strain, GK32, and generated mutant GK32/pOX15Y420F, colonies of GK32 showed virtually no *cbb*₃ oxidase activity (NADI⁻ phenotype-no blue color formed within 30min) (Figure 3.29a), on the other hand, GK32/pOX15 colonies showed NADI⁺ phenotype (Figure 3.29b) as expected. The generated mutant colonies

were showed NAD⁺ phenotype like negative control strain, GK32. NAD⁺ staining phenotype of GK32/pOX15Y420F mutant reveals that Tyrosine 420 is a critical amino acid for enzyme activity.

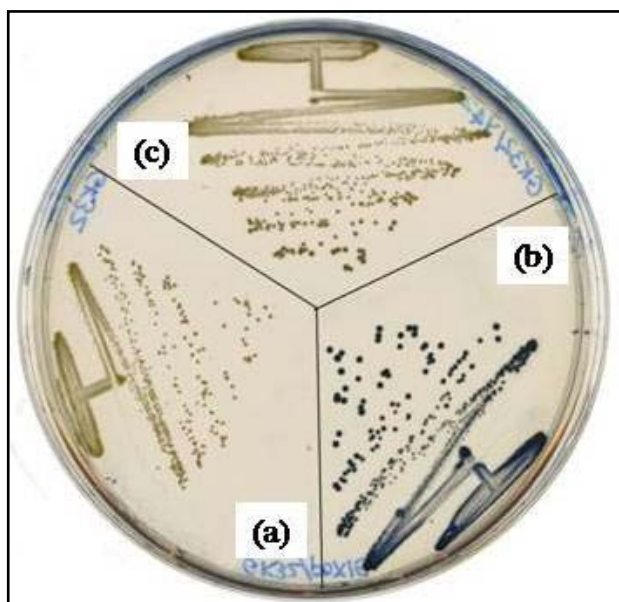


Figure 3.29 NAD⁺ stained plate of GK32/pOX15Y420F mutant (a) represents NAD⁺ stained *R. capsulatus* GK32 strain as negative control with NAD⁺ phenotype; (b) represents NAD⁺ stained GK32/pOX15 strain as positive control with NAD⁺ phenotype; (c) represents NAD⁺ stained GK32/pOX15Y420F mutant with NAD⁺ phenotype.

3.4.7. Re-sequencing of Mutant GK32/pOX15Y420F

Before starting the characterization experiments, plasmid DNA from GK32/pOX15Y420F mutant strain was isolated and sequenced at DNA Sequencing Facility of University of Pennsylvania to make sure that each plasmid carries the desired mutation. Sequences obtained from plasmid DNAs were compared with the sequence of the *cbb₃* oxidase from NCBI Gene Databank and found that GK32/pOX15Y420F DNA carries the desired mutation (AAG) and there is no mutation other than desired one (Figure 3.30).

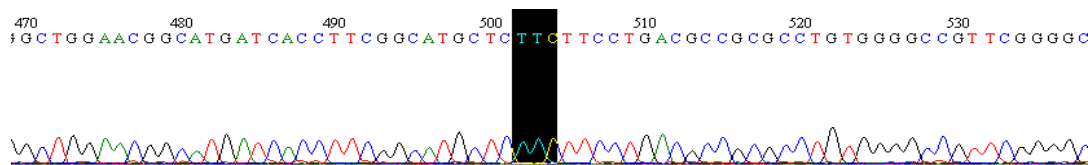


Figure 3.30 The partial sequence of GK32/pOX15Y420F transformant. The TAC codon was converted to AAG codon.

3.4.8. Detection of Subunit I in GK32/pOX15Y420F Mutant by Western Blot Analysis

For CcoN immunodetection, chromatophore membrane proteins were electroblotted onto Immobilon-P membranes and immunoglobulins bound to crossreacting *R. capsulatus* proteins were detected with alkaline phosphates-conjugated monoclonal anti-rabbit secondary antibodies. Western blot analyses revealed that CcoN was detected in MT1131 (Figure 3.31; Line 4) and GK32/pOX15 (Figure 3.31; Line 2) and also, was absent in GK32 (Figure 3.31; Line 3) as expected. The western analysis of GK32/pOX15Y420F mutant showed that, this mutant lacked CcoN of the cyt *cbb*₃ oxidase (Figure 3.31; Line 1).

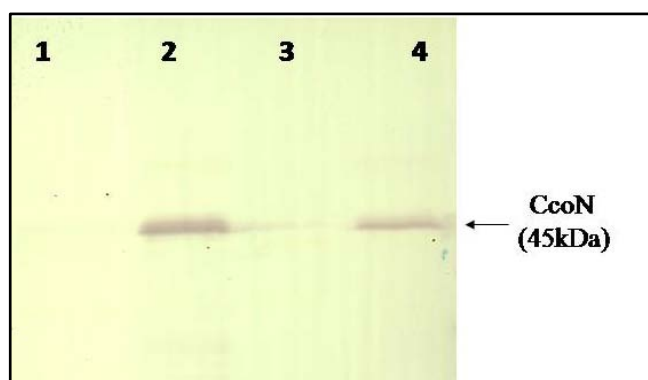


Figure 3.31 Western blot analyses of GK32/pOX15Y420F. Western blot analyses with anti-CcoN (subunit I) antibodies. After SDS-PAGE (10 µg of membrane proteins per lane) electrophoretically transferred onto an Immobilon-P membrane. Line 1 contains GK32/pOX15Y420F; Line 2 contains GK32/pOX15, as positive complemented mutant; Line 3 contains GK32, as negative control; Line 4 contains wild-type, MT1131.

3.4.9. Cytochrome Profiles of GK32/pOX15Y420F Mutant

The desired mutant GK32/pOX15Y420F together with wild type, MT1131, positive control strain, GK32/pOX15, and negative control strain, GK32, were analyzed for their *c*-type cyt content by using TMBZ/SDS-PAGE analysis and Coomassie Brilliant Blue staining. After TMBZ staining of the SDS-PAGE, it was found that subunits of the GK32/pOX15Y420F mutant has no CcoP and CcoO subunits of *cbb*₃ oxidase (Figure 3.32, Line 1), which is similar to negative control strain, GK32. Coomassie Brilliant Blue staining of the gel was also support that TMBZ staining results (Figure 3.33). Together with western blotting results, it has been showed that GK32/pOX15Y420F mutant lacks all subunits of the cyt *cbb*₃ oxidase therefore, it could be said that Tyrosine residue at position of 420 is important for cyt *cbb*₃-type oxidase assembly.

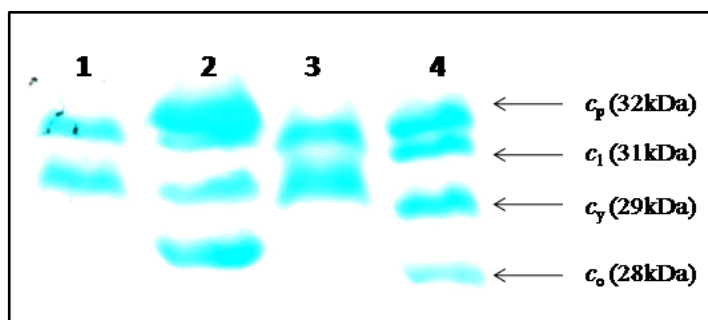


Figure 3.32 Cytochrome *c* profile of GK32/pOX15Y420F mutant grown in enriched MPYE medium under respiratory conditions. Approximately 50 µg of protein was loaded in each lane of a 16.5% SDS polyacrylamide gel. After electrophoresis, *c*-type cyts were detected by TMBZ staining. Line 1 contains GK32/pOX15Y420F; Line 2 contains GK32/pOX15, as positive complemented mutant; Line 3 contains GK32, as negative control; Line 4 contains wild-type, MT1131. cyt *c*_p and *c*_o are the subunits II and III of cyt *cbb*₃ oxidase, and cyts *c*₁ and *c*_y correspond to the cyt *c*₁ subunit of the *bc*₁ complex and the membrane-attached electron carrier *c*_y, respectively

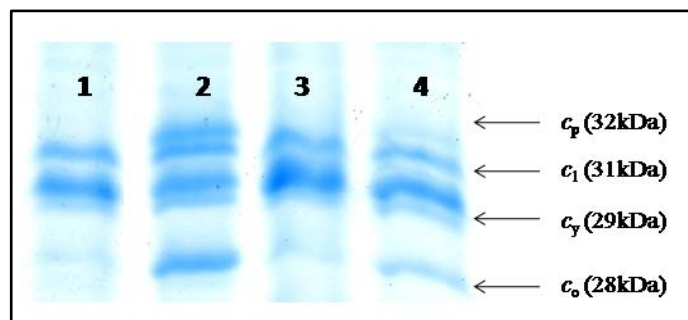


Figure 3.33 Coomassie Brilliant Blue staining of GK32/pOX15Y420F mutant. Line 1 contains GK32/pOX15Y420F; Line 2 contains GK32/pOX15, as positive complemented mutant; Line 3 contains GK32, as negative control; Line 4 contains wild-type, MT1131. cyt c_p and c_o are the subunits II and III of cyt cbb_3 oxidase, and cyts c_1 and c_y correspond to the cyt c_1 subunit of the bc_1 complex and the membrane-attached electron carrier cy , respectively.

3.4.10. Cytochrome c Oxidase Activity of GK32/pOX15Y420F Mutant

The oxygen consumption rate of chromatophore membranes of MT1131 (wild type), GK32/pOX15, GK32 and GK32/pOX15Y420F mutant were measured spectrophotometrically by monitoring the oxidation of reduced horse heart cyt c . The measurements of cbb_3 oxidase activity results indicate that the GK32/pOX15Y420F mutant revealed 8.1% activity of cbb_3 oxidase. Characterization data revealed that Tyrosine residue at position of 420 is important for the structural characteristic of cyt cbb_3 -type oxidase from *R. capsulatus*.

3.5. Construction and Characterization of GK32/pOX15F480A Mutant

3.5.1. Sequencing of pGY*F480A Mutant

Plasmid DNA from pGY*F480A mutant was isolated and sequenced. Obtained sequence for plasmid DNA of pGY*F480A was compared with the sequence of the cbb_3 oxidase from NCBI gene data bank. Sequence results showed that, pGY*F480A

sequence has the desired GCC (reverse complement: CGG) mutation (Figure 3.34). and there is no other mutation than desired one created between restriction enzyme sites were detected.

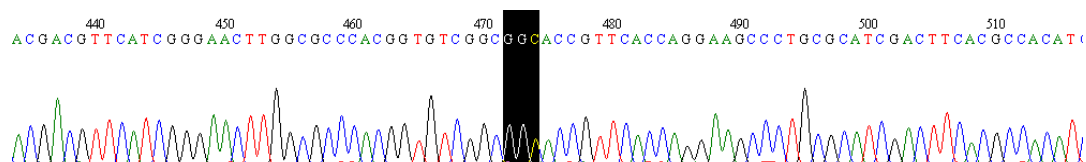


Figure 3.34 The partial sequence of pGY*F480A transformant. The TTC codon was converted to GCC codon which is labeled (as reverse complement CGG).

3.5.2. Replacement of the 0.6 kbp NO' Fragment of pGY with that of pGY*F480A Mutant

To replace 0.6 kbp fragment of pGY with that of pGY*F480A, DNAs from pGY and pGY*F480A were isolated and digested with *Sph*I and *Sac*I restriction enzymes. *Sph*I and *Sac*I digested pGY DNA was dephosphorylated by shrimp alkaline protease (SAP) to prevent ligation of vectors with each other. The *Sph*I and *Sac*I digested DNAs, pGY, and pGY*F480A were loaded on 1% agarose gel. Resulting fragments, 0.6 kbp, and 5.1 kbp, were separated (Figure 3.35). Then 5.1 kbp fragment from pGY and 0.6 kbp fragment from pGY*F480A were isolated from agarose gel (Figure 3.36 and Figure 3.37). The amounts of the isolated insert and vector were determined by using gel electrophoresis (Figure 3.38). After that, 0.6 kbp fragment from pGY*F480A was inserted into 5.1 kbp fragment from pGY and transformed to XL1-Blue competent cells. Four different transformants were selected on the basis of their size (5.7 kbp) and ampicillin resistance. Plasmid DNAs from four different plasmids were isolated and sequenced to ensure the each transformant

has the desired mutation. After sequencing the transformants the new clones with desired mutation were saved and designated as XL1-Blue/pGYF480A.

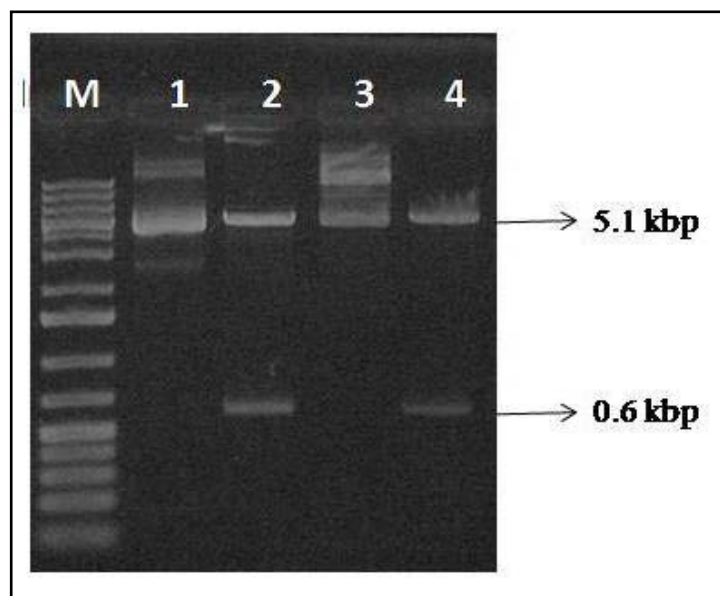


Figure 3.35 Digestion of pGY and pGY*F480A DNAs with *Sph*I and *Sac*I Res. Line 1 contains uncut pGY DNA as control; Line 2 contains *Sph*I and *Sac*I digested dephosphorylated pGY DNA with resulting fragments, 5.1 kbp and 0.6 kbp; Line 3 contains uncut pGY*F480A DNA as control; Line 4 contains *Sph*I and *Sac*I digested pGY*F480A DNA with resulting fragments, 5.1 kbp and 0.6 kbp. Marker is designated as M.

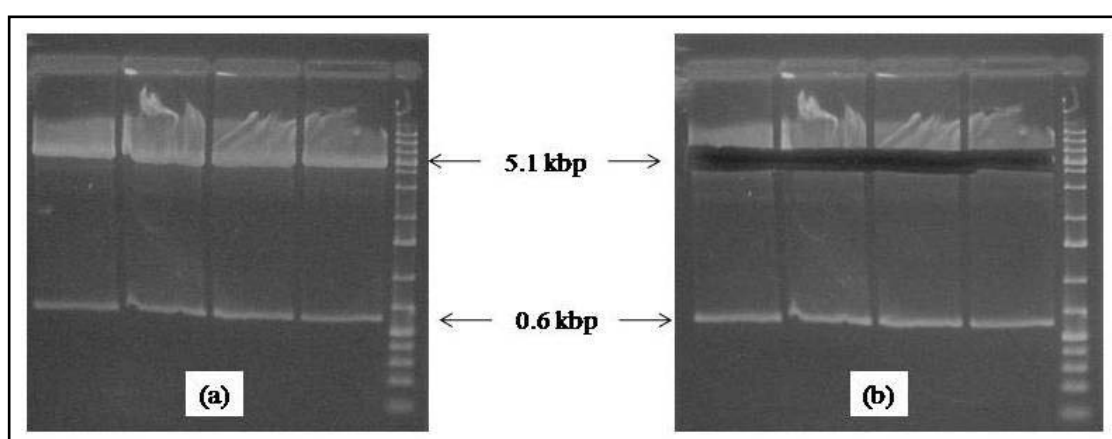


Figure 3.36 Isolation and separation of *Sph*I and *Sac*I digested dephosphorylated pGY DNA. **(a)** Separation of 5.1 kbp and 0.6 kbp fragments from *Sph*I and *Sac*I digested pGY DNA. **(b)** Isolation of 5.1 kbp fragment from 1% agarose gel

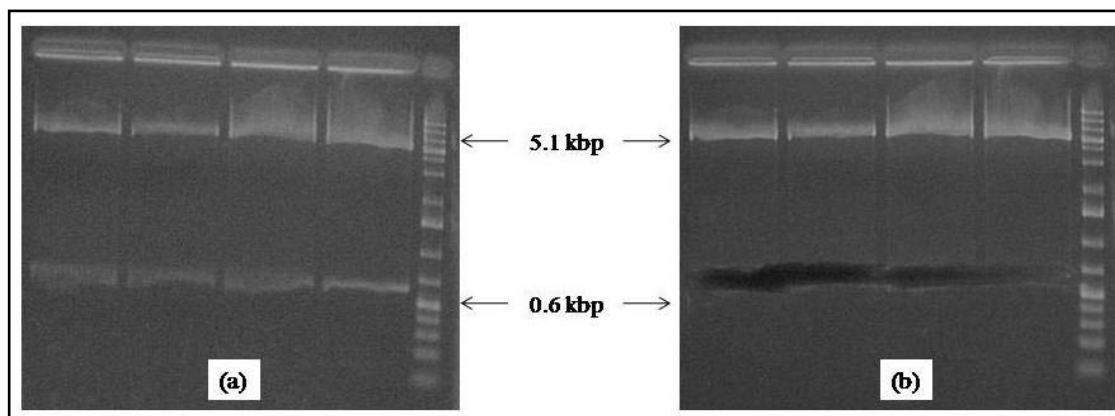


Figure 3.37 Isolation and separation of *SphI* and *SacI* digested pGY*F480A DNA. **(a)** Separation of 5.1 kbp and 0.6 kbp fragments from *SphI* and *SacI* digested pGY*F480A DNA. **(b)** Isolation of 0.6 kbp fragment from 1% agarose gel.

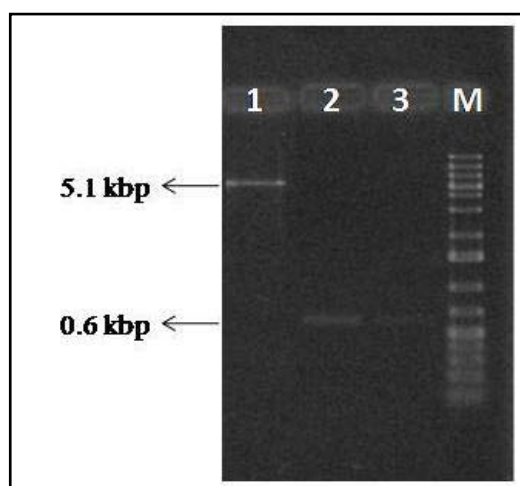


Figure 3.38 Determination of the amount of dephosphorylated pGY vector DNA and insert DNA from pGY*F480A. Determination of the amount of 5.1 kbp fragment of *SphI* and *SacI* digested dephosphorylated pGY vector DNA and 0.6 kbp insert DNA from pGY*F480A. Line 1 contains the 5.1 kbp fragment of pGY vector DNA (20 ng/μl); Line 2 and 3 contain 0.6 kbp fragment of insert ((10 ng/μl) and (5 ng/μl), respectively) from pGY*F480A. Marker is designated as M.

3.5.3. Replacement of the 2.8 kbp NO' Fragment of pOX15 with that of pGYF480A Mutant

For the preparation of 15.3 kbp pOX15F480A DNA, pGYF480A DNA was digested by *Alw441* and *XhoI* restriction enzymes. pOX12 DNA was digested with *XhoI* restriction enzyme and dephosphorylated by shrimp alkaline phosphatase (SAP) to prevent self ligation of the vector. Digested samples were loaded on 1% agarose gel and 12.5 kbp fragment from pOX12, and 2.8 kbp, 1.3 kbp, 0.9 kbp and 0.7 kbp fragments from pGYF480A were separated (Figure 3.39). Linear pOX12 DNA (12.5 kbp) and 2.8 kbp fragment from pGYF480A were isolated from gel (Figure 3.40 and Figure 3.41). Before ligation, the amounts of vector and insert were determined by loading of 1 µl DNAs of each on 1% agarose gel (Figure 3.42). Finally, ligation process was handled by inserting the 2.8 kbp fragment from pGYF480A into dephosphorylated linear pOX12 vector and transformed into DH5α competent cells. Four different transformants were selected based on clone size, 15.3 kbp and tetracycline resistance. The new clones were designated as DH5α/pOX15*F480A before orientation check of the insert.

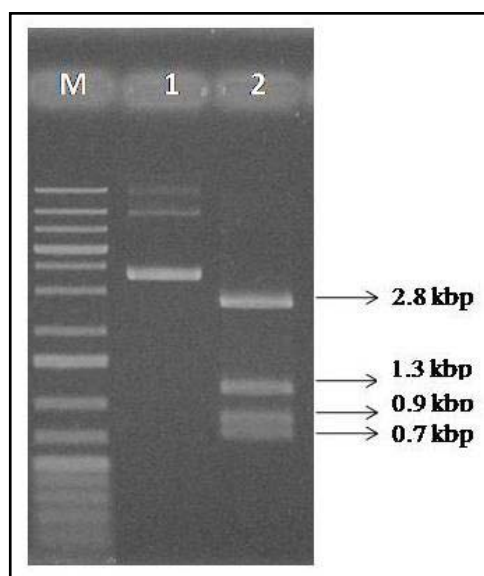


Figure 3.39 Separation of *Alw*441 and *Xho*I digested pGYF480A DNA. Line 1 contains uncut pGYF480A DNA; Line 2 contains *Alw*441 and *Xho*I digested pGYF480A DNA with resulting fragments, 8 kbp, 1.3 kbp, 0.9 kbp and 0.7 kbp; Marker is designated as M.

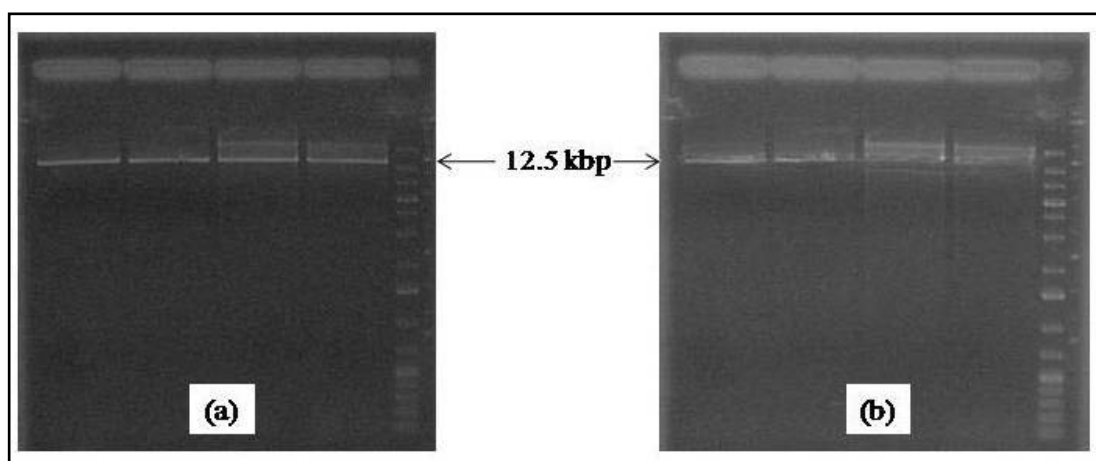


Figure 3.40 Isolation and separation of *Xho*I pOX12 DNA. **(a)** Separation of dephosphorylated 12.5 kbp linear pOX12 DNA. **(b)** Isolation of dephosphorylated 12.5 kbp fragment from 1% agarose gel.

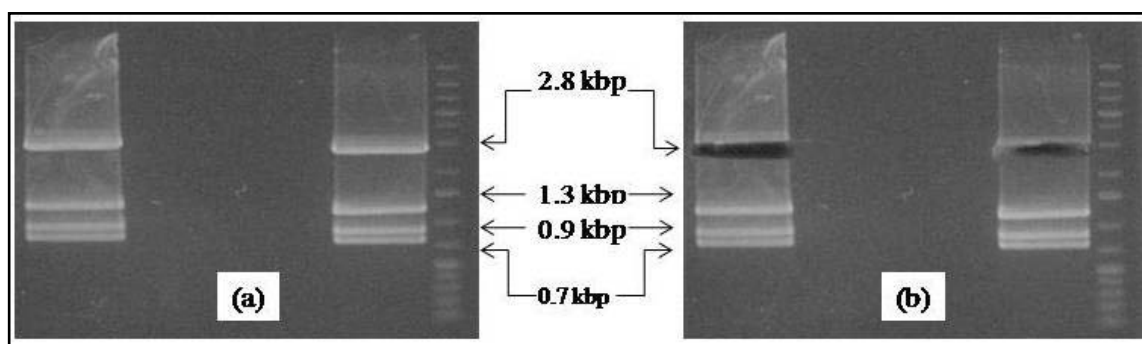


Figure 3.41 Isolation and separation of *Alw*441 and *Xho*I digested pGYF480A DNA. **(a)** Separation of *Alw*441 and *Xho*I digested pGYF480ADNA with resulting fragments, 2.8 kbp, 1.3 kbp, 0.9 kbp and 0.7 kbp. **(b)** Isolation of 2.8kbp fragment from 1% agarose gel

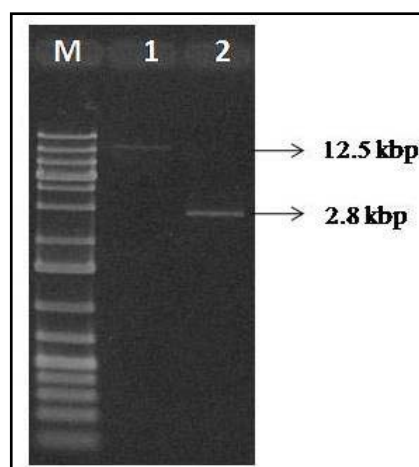


Figure 3.42 Determination of the amount of dephosphorylated pOX12 vector DNA and insert DNA pGYF480A DNA. Determination of the amount of 12.5 kbp fragment of *Xho*I digested and dephosphorylated pOX12 vector DNA and 2.8 kbp insert DNA pGYF480A DNA. Line 1 contains the 12.5 kbp fragment of dephosphorylated pOX12 vector DNA (10 ng/μl); Line contains 2.8 kbp fragment of insert (30 ng/μl) from pGYF480A; Marker is designated as M.

3.5.4. Control of the 2.8 kbp Insert Orientation of pOX15*F480A Mutant

To select the clone that has correct oriented insert, four different plasmids DNAs of pOX15*F480A were isolated from transformants and digested with *Hind*III RE. After digestion of pOX15*F480A DNAs from transformants, it was found that one of them is correctly oriented (Figure 3.43, Line 5) and the others were wrong oriented (Figure 3.43 Lines 3, 4 and 6). The new clones that have correct oriented mutated insert were kept at -80°C and named as DH5 α /pOX15F480A.

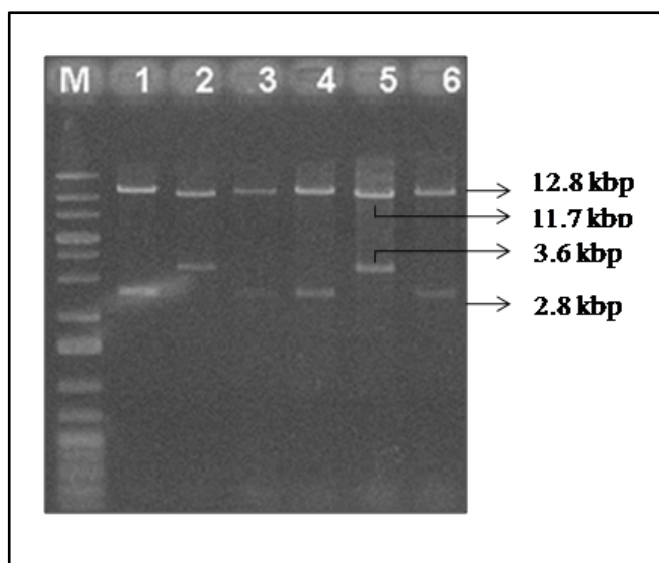


Figure 3.43 *Hind*III Digestion of DH5 α /pOX15*F480A transformants. Line 1 contains control of wrong orientation-2.8 kbp and 12.5 kbp fragments; Line 2 contains control of correct orientation-3.6 kbp and 11.7 kbp fragments; Line 3, 4 and 6 contain *Hind*III digested pOX15*F480A DNA-wrong oriented; Line 5 contain *Hind*III digested pOX15*F480A DNA-correct oriented; Marker is designated as M.

3.5.5. Transformation of pOX15F480A to *R. capsulatus* By Triparental Mating

The pOX15F480A plasmid DNA that is in DH5 α strains of *E. coli* was transferred into GK32 strain of *R. capsulatus* in the presence of HB101/pRK2013 helper cells. Transconjugants were selected based on tetracycline, kanamycin resistance and named as GK32/pOX15F480A were selected and kept for further analysis at -80°C.

3.5.6. Determination of *cbb*₃ Oxidase Phenotype Profile of GK32/pOX15F480A Mutant with NADI Staining

NADI staining was performed with the whole cells of complemented control mutant, GK32/pOX15, GK32 and mutant GK32/pOX15F480A. Results revealed that, GK32/pOX15 had *cbb*₃ oxidase activity which causes NADI⁺ phenotype (Figure 3.44a). However colonies of GK32 had virtually no *cbb*₃ oxidase activity that resulted as NADI⁻ phenotype (Figure 3.44b). The *cbb*₃ oxidase activity was detected in the mutant strain GK32/pOX15F480A however it exhibited slow NADI⁺ phenotype which required an incubation time longer than 2 min to turn blue (Fig 3.44c). NADI staining phenotype of GK32/pOX15F480A mutant reveals that Phenylalanine 480 amino acid is not so critical for enzyme activity might have fewer roles in enzyme activity.

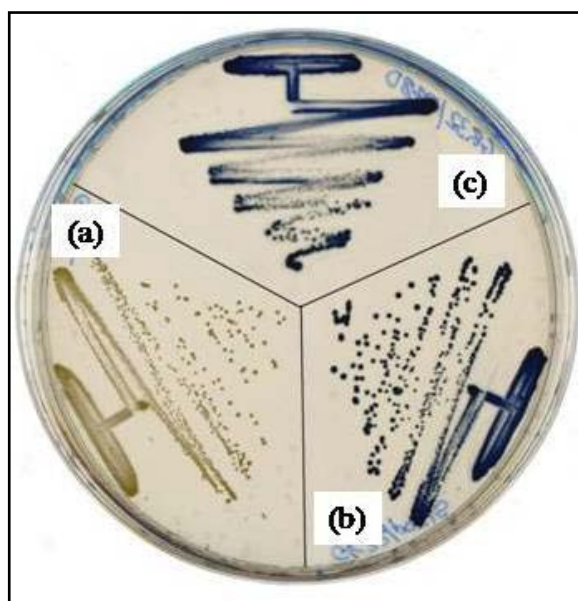


Figure 3.44 NADH stained plate of GK32/pOX15F480A mutant. (a) represents NADH stained GK32/pOX15 strain as positive control with NADH⁺ phenotype; (b) represents NADH stained *R. capsulatus* GK32 strain as negative control with NADH⁻ phenotype; (c) represents NADH stained GK32/pOX15F480A mutant with NADH⁺ slow phenotype

3.5.7. Re-Sequencing of the Mutant GK32/pOX15F480A

Before starting the characterization and pumping experiments plasmid DNA from GK32/pOX15F480A mutant strain was isolated and sequenced at DNA Sequencing Facility of University of Pennsylvania to make sure that plasmid carries the desired mutation. Sequences obtained from plasmid DNAs were compared with the sequence of the *cbb₃* oxidase from NCBI Gene Databank and found that TTC codon was successfully substituted to GCC codon in GK32/pOX15F480A DNA and there is no mutation other than desired one (Figure 3.45)

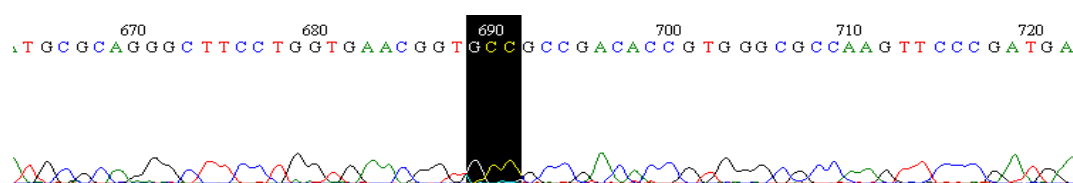


Figure 3.45 The partial sequence of GK32/pOX15F480A transformant. The TTC codon was converted to GCC codon.

3.5.8. Detection of CcoN in GK32/pOX15F480A Mutant by Western Blot Analysis

Western blot analysis for CcoN, chromatophore membrane proteins were electroblotted onto Immobilon-P membranes. Results revealed that CcoN was detected in MT1131 (Figure 3.46; Line 4) and GK32/pOX15 (Figure 3.46; Line 2) and also, was absent in GK32 (Figure 3.46; Line 3) as expected. CcoN was also detected in the chromatophore membranes of GK32/pOX15F480A, similar to wild-type and positive control mutant (Figure 3.46, Line 1).

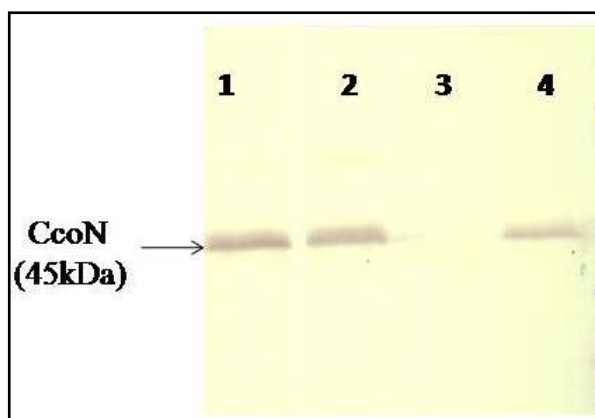


Figure 3.46 Western blot analyses of GK32/pOX15F480A mutant. Western blot analyses with anti-CcoN (subunit I) antibodies. After SDS-PAGE (10 μ g of membrane proteins per lane) and electrophoretically transferred onto an Immobilon-P membrane. Line 1 contains GK32/pOX15F480A; Line 2 contains GK32/pOX15, as positive complemented mutant; Line 3 contains GK32, as negative control; Line 4 contains wild-type, MT1131.

3.5.9. Cytochrome Profiles of GK32/pOX15F480A Mutant

After TMBZ staining of the chromatophore membranes MT1131, GK32/pOX15, GK32 and mutant GK32/pOX15F480A loaded SDS-PAGE gel; it was found that subunits of the GK32/pOX15F480A mutant are almost the same with those of the controls, MT1131 and GK32/pOX15 (Figure 3.47). Alternatively

Coomassie Brilliant Blue staining of the gel also supported the TMBZ staining results (Figure 3.48). This profile together with western blotting results showed that Phenylalanine at position 480 is not significant for assembly of the *cyt cbb₃*-type oxidase.

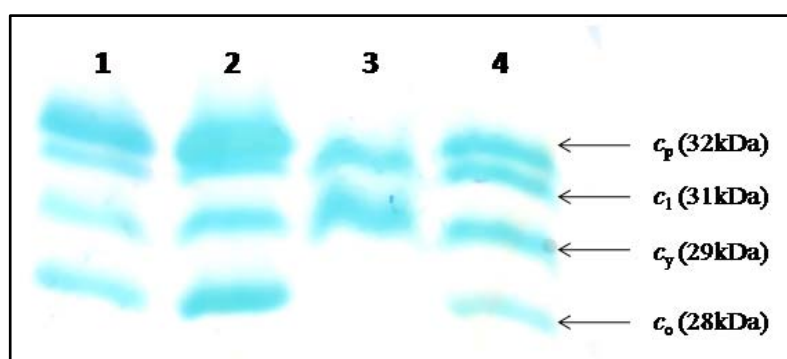


Figure 3.47 Cytochrome *c* profile of GK32/pOX15F480A mutant grown in enriched MPYE medium under respiratory conditions. Approximately 50 μ g of protein was loaded in each lane of a 16.5% SDS polyacrylamide gel. After electrophoresis, *c*-type cyts were detected by TMBZ staining. Line 1 contains GK32/pOX15F480A; Line 2 contains GK32/pOX15, as positive complemented mutant; Line 3 contains GK32, as negative control; Line 4 contains wild-type, MT1131. *cyt c_p* and *c_o* are the subunits II and III of *cyt cbb₃* oxidase, and *cyts c₁* and *c_y* correspond to the *cyt c₁* subunit of the *bc₁* complex and the membrane-attached electron carrier *c_y*, respectively.

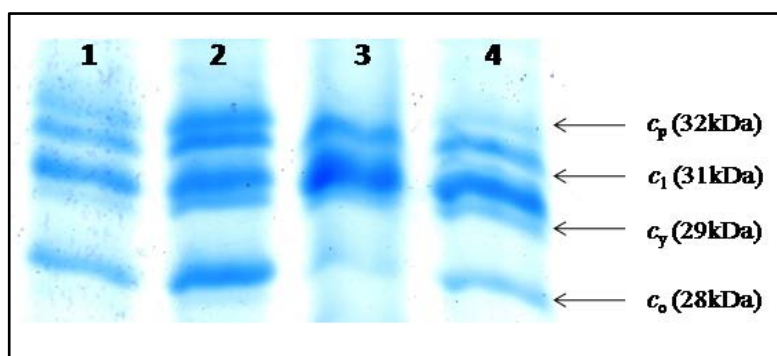


Figure 3.48 Coomassie Brilliant Blue staining of GK32/pOX15Y420F mutant. Line 1 contains GK32/pOX15F480A; Line 2 contains GK32/pOX15, as positive complemented mutant; Line 3 contains GK32, as negative control; Line 4 contains wild-type, MT1131. *cyt c_p* and *c_o* are the subunits II and III of *cyt cbb₃* oxidase, and *cyts c₁* and *c_y* correspond to the *cyt c₁* subunit of the *bc₁* complex and the membrane-attached electron carrier *c_y*, respectively.

3.5.10. Cytochrome *c* Oxidase Activity of GK32/pOX15F480A Mutant

Cytochrome *c* oxidase activity of chromatophore membranes from MT1131, GK32/pOX15, GK32 and mutant GK32/pOX15F480A were measured spectrophotometrically by monitoring the oxidation of reduced horse heart cyt *c* in the presence of TMPD as an electron donor. The measurements of *cbb*₃ oxidase activity results indicate that GK32/pOX15F480A mutant showed 71,8 % activity which conforms its slow positive cyt *c* oxidase phenotype resulted from NADI Staining.

3.6. Construction and Proton Pumping of KZ101/pOX15F480A Mutant

3.6.1. Transformation of pOX15F480A to KZ101 Strain of *R. capsulatus* by Triparental Mating

Rhodobacter capsulatus is good candidate for proton translocation experiments because of having one quinol oxidase and one *cbb*₃-type oxidase as terminal oxidase. To understand the effects of mutation on proton translocation, the mutation at the position of Phenylalanine 480 is also good candidate in all mutants. Characterization experiments revealed that this mutation had no affect on assembly and caused decrease in activity. As the *cbb*₃-type oxidases also pump protons, this residue might have role in proton pumping. To detect the effect of mutation on proton translocation a new and unique system was needed which lacks all kind of oxidases. For this purpose KZ101 strain of *R. capsulatus* which lacks both quinol and *cbb*₃-type oxidases was chosen. To measure the proton translocation pOX15, pRK415 and

pOX15F480A plasmid DNAs were transferred into KZ101 strain of *R. capsulatus* in the presence of HB101/pRK2013 helper cells by triparental mating. Selected transconjugants were named as KZ101/pOX15, KZ101/pRK415 and KZ101/pOX15F480A and kept for further analysis at -80°C.

3.6.2. NADI staining of the KZ101 strains

Before starting the proton pumping experiments, to ensure that each plasmid was transferred successfully to KZ101 strain of *R. capsulatus* and the enzyme is still active quantitative analysis of cyt *cbb*₃ oxidase activity was performed by NADI Staining with the photosynthetically grown strains. As the strains grown under PS conditions, colonies have green color, after performing NADI staining color change upon active cyt *cbb*₃ oxidase was not clear. However, it was observed that, if the strain had an active cyt *cbb*₃ oxidase, colonies grown on Med A medium turned into deep green and this situation was accepted as NADI⁺. NADI staining results of four transconjugants from each KZ101/pOX15, KZ101/pRK415 and KZ101/pOX15 strains were indicated that KZ101/pOX15 transconjugants have NADI⁺ phenotype (Figure 3.49a) and KZ101/pOX15F480A transconjugants have slow NADI⁺ phenotype (Figure 3.49b) while KZ101/pRK415 transconjugants have NADI⁻ phenotype (Figure 3.49c).

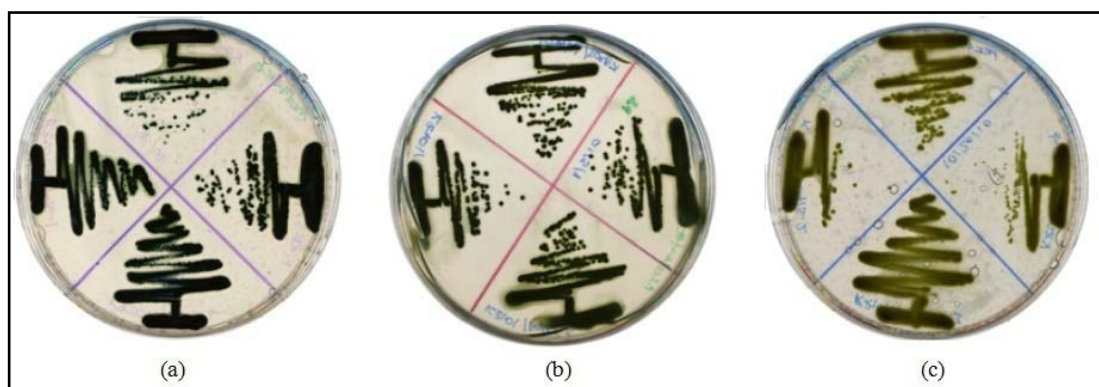


Figure 3.49 NADH stained plates of KZ101transconjugants. **(a)**represents NADH stained *R. capsulatus* KZ101/pOX15 transconjugants with NADH⁺ phenotype **(b)** represents NADH stained *R. capsulatus* KZ101/pOX15F480A transconjugants with slow NADH⁺ phenotype **(c)** represents NADH *R. capsulatus* KZ101/pRK415 transconjugants with NADH⁻ phenotype

3.6.3. Whole Cell Proton Pumping Profile of KZ101/pOX15F480A Mutant

Whole cell proton pumping measurements of the mutant KZ101/pOX15F480A and the complement mutant KZ101/pOX15 were determined by the change in pH, which was monitored with a sensitive pH electrode. KZ101 strain of *R. capsulatus*, *cyt c* and quinol oxidase knock out strain, allowed to understand the effect of mutation on proton translocation in *cbb₃* type of oxidase. Not only using the double knocked strain, but also TMPD alone was used as the only reductant. Myxothiazol was also added to ensure that no electrons could be transferred through *bc₁* complex to the oxygen reductase. *cbb₃*-type oxidase was shown to be cyanide sensitive at concentrations less than 100 μ M. Positive control strain KZ101/pOX15 was shown to translocate protons 0.5-0.6 H⁺/e (Figure 3. 50) whereas in negative control KZ101/pRK415 was not shown to translocate protons (Figure 3.51) as expected. The mutant KZ101/pOX15F480A was showed similar proton translocation characteristics like negative control strain which means it was not able to translocate protons

(Figure 3.52). Thus it has been shown that the phenylalanine residue at the position of 480 might have role in the proton translocation.

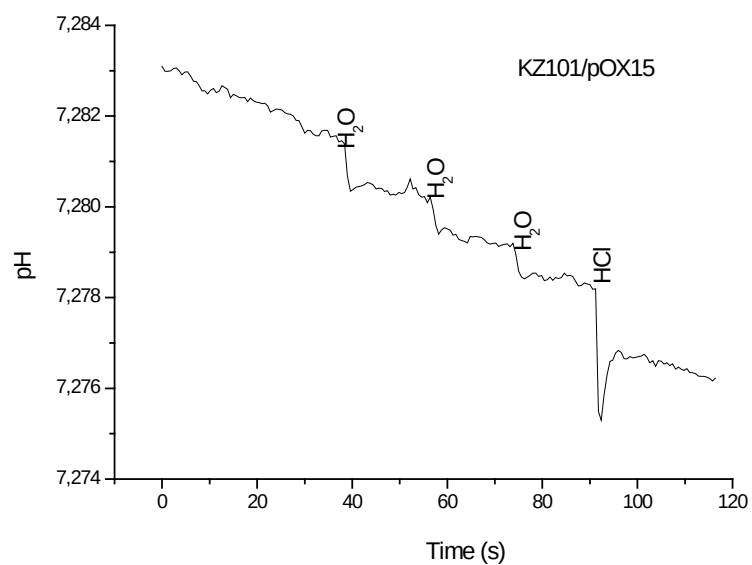


Figure 3.50 Oxygen respiration by KZ101/pOX15. Change in pH upon addition of 10 µl O₂ by KZ101/pOX15. pH changes caused by oxygen addition was calibrated by adding the same amount of HCl.

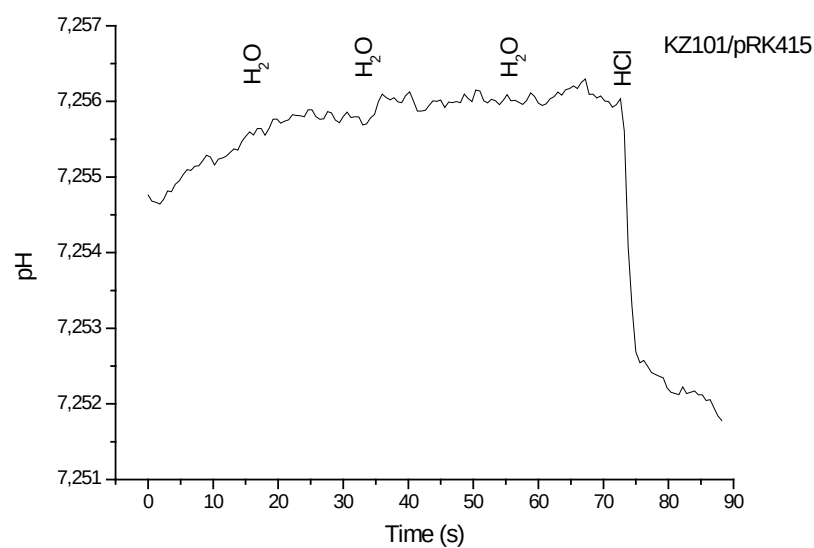


Figure 3.51 Oxygen respiration by KZ101/pRK415. Change in pH upon addition of 10 µl O₂ by KZ101/pRK415. pH changes caused by oxygen addition was calibrated by adding the same amount of HCl.

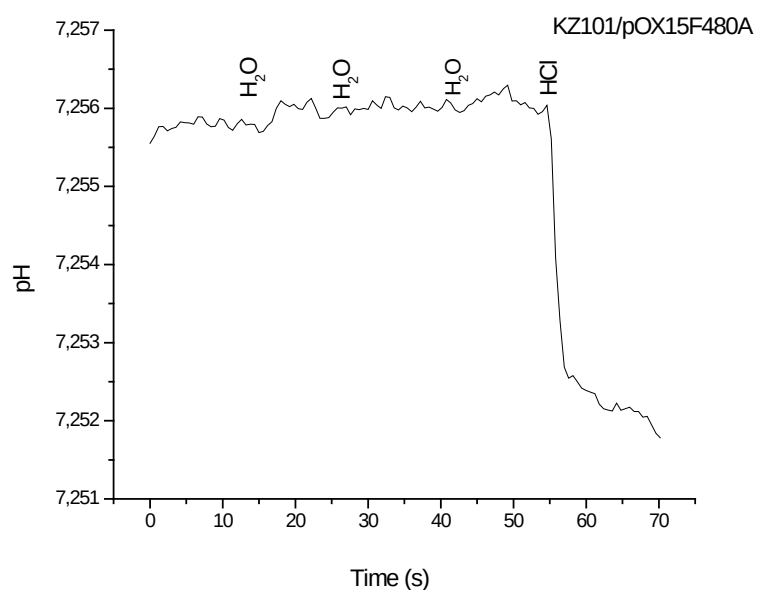


Figure 3.52 Oxygen respiration by KZ101/pOX15F480A. Change in pH upon addition of 10 µl O₂ by KZ101/pOX15F480A. pH changes caused by oxygen addition was calibrated by adding the same amount of HCl.

3.7. Construction of His-tagged Plasmid for the Isolation of *cbb₃* from *R. capsulatus*

3.7.1. Construction of pGY2

A part of *orf277*, *ccoNOQP* operon of the *cbb₃*-type oxidase and small part of *ccoG* has already been cloned into pRK415(10.5 kb) expression vector and named aspCcoNHisOQP by Grzegorz Pawlik(unpublished data). Similar to pOX15 DNA this DNA is heat sensitive and delicate thus is not suitable to use as a template DNA in site-directed mutagenesis procedure. To overcome this problem a new template DNA, pGY2, was constructed. For this purpose, pCcoNHisOQP DNA was digested with *KpnI* and *SpeI* restriction enzymes and resulting fragments 10.7 kbp and 4.6 kbp were separated on agarose gel and fragments were isolated from agarose gel to use as an insert. Also, pBluescript DNA was digested with same restriction enzymes to prepare vector and separated agarose gel and ~2.8 kbp fragment was isolated from agarose gel (Figure 3.53). 4.6 kbp fragment was inserted into 2.8 kbp pBluescript vector by ligation reaction and then transformed chemical competent cells. Transformants were selected by using Blue/White Colony Assay and the clone, 7.4 kbp in size was obtained and designated as TOP10/pGY2(Figure 3.54)

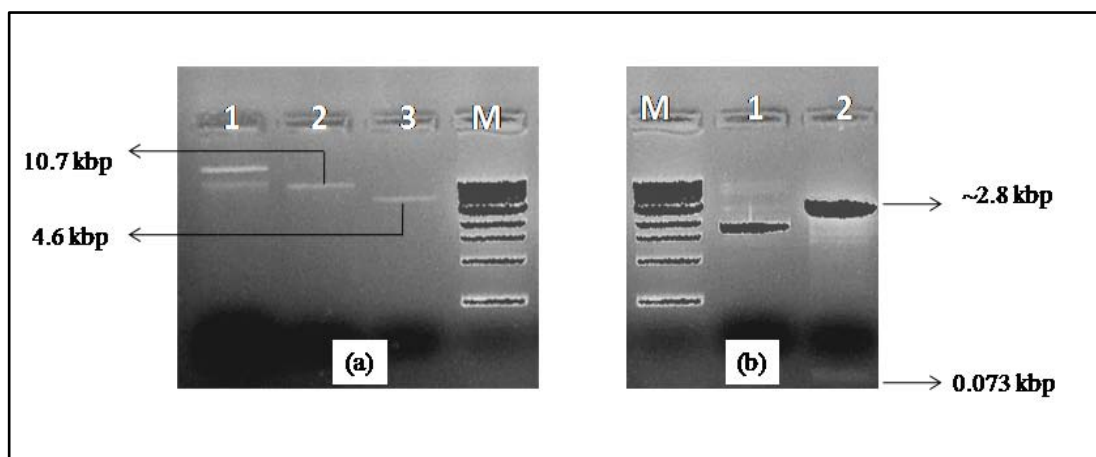


Figure 3.53 Digestion of pBluescript and pCcoNHisoQP DNAs with *KpnI* and *SpeI* REs. **(a)** Digestion of pCcoNHisoQP DNA with *KpnI* and *SpeI* restriction enzymes. Line 1 contains uncut pCcoNHisoQP DNA; Line 2 contains, 10.7 kbp pCcoNHisoQP; Line 3 4.6 kbp insert. **(b)** Digestion of pBluescript DNA with *KpnI* and *SpeI* restriction enzymes. Line 1 contains uncut pBluescript DNA as control; Line 2 contains, *KpnI* and *SpeI* digested pBluescript DNA with resulting fragments, 2.827 kbp and 0.073 kbp. Marker is designated as M.

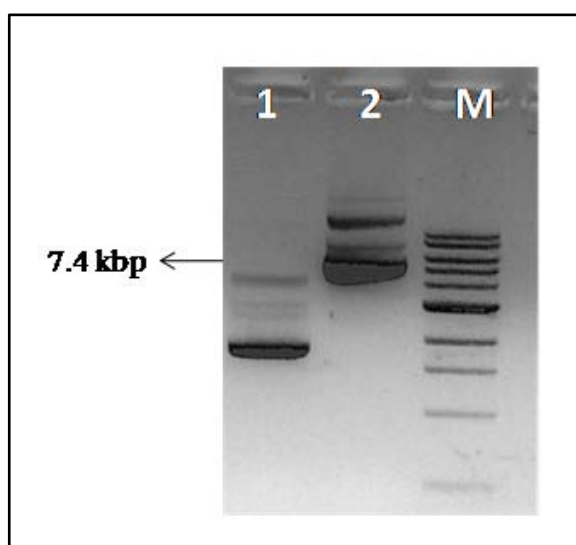


Figure 3.54 Selection of pGY2 plasmid DNA depending on size (7.4.kbp). Line 1 contains uncut pBluescript DNA as control; Line 2 contains, 7.4 kbp pGY2 DNA

3.7.2. Addition of His-Tag to the C- terminus of *ccoP* by PCR Based Site-Directed Mutagenesis

Plasmid DNAs from both TOP10/pGY2CcoNOQP4HIS and TOP10/pGY2CcoNOQP8HIS were isolated and sequenced to verify the histidine codons. Obtained sequence was compared with the sequence of the *cbb₃* oxidase from NCBI gene data bank (*Rhodobacter capsulatus* SB 1003 chromosome-gi|294675557:1226693-1228291). It was found that the sequence of pGY2CcoNOQP4HIS had desired first four histidine codons (CATCACCATCAC) (Figure 3.55) and pGY2CcoNOQP8HIS had eight histidine codons (CATCACCATCACCATCACCATCAC) at the C-terminus of the *ccoP* just before its stop codon (Figure 3.56), and no other mutation was detected.

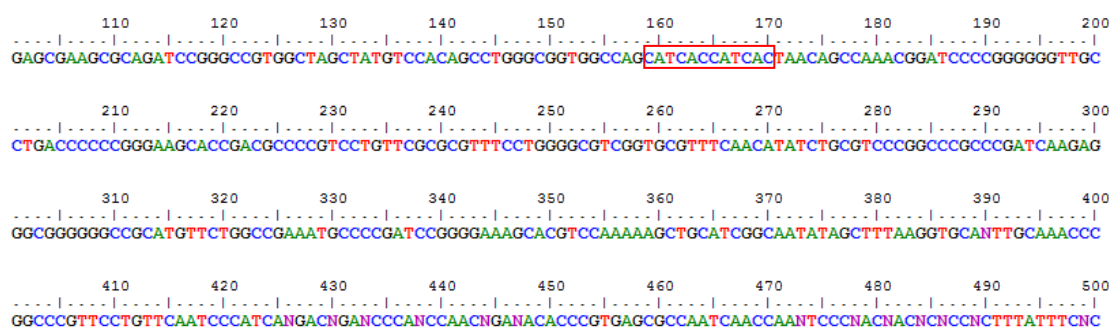


Figure 3.55 Sequence of pGY2CcoNOQP4HIS. Four histidine codons (CATCACCATCAC) was detected in the sequence of pGY2CcoNOQP4HIS.

```

      110      120      130      140      150      160      170      180      190      200
      |-----|-----|-----|-----|-----|-----|-----|-----|-----|
GAGCAAGCGCAGATCCGGGCCGTGGCTAGCTATGTCCACAGCCTGGGCGGTGGCCAGCATCACCATCACCATCACCATCAC
      210      220      230      240      250      260      270      280      290      300
      |-----|-----|-----|-----|-----|-----|-----|-----|-----|
CGGGGGGTTGCCTGACCCCCCGGAAGCACCAGCGCCCCGTCTGTTCGCGCTTTCCTGGGGCGTCGGTGCGTTTCAACATATCTGCGTCCCGGCCGCG
      310      320      330      340      350      360      370      380      390      400
      |-----|-----|-----|-----|-----|-----|-----|-----|-----|
CCGATCAAGAGGGGCGGGGGCCGCATGTCTGGCCGAAATGCCCGGATCCGGGAAAGCACGTCCAAAAAGCTGCATCGGCAATATAGCTTTAAGGTGCA
      410      420      430      440      450      460      470      480      490      500
      |-----|-----|-----|-----|-----|-----|-----|-----|-----|
GTTGCAAAACCCGCCCGTTCTGTTCATCCCATCAGGACGGAGCCCAAGCCAGGAGACCCGTGAGCGCCAATCAACCAAGTCCCGACGACGCGCG

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Figure 3.56 Sequence of pGY2CcoNOQP8HIS. Eight histidine codons (CATCACCATCACCATCACCATCAC) were detected in the sequence of pGY2CcoNOQP8HIS

3.7.3. Replacement of the 4.6 kbp NOQP8His Fragment of pGY2ccoNOQP8HIS with that of pCcoNHisOQP

To prepare 15.3 kbp pCcoNHisOQP8HIS DNA, plasmid DNAs of pGY2CcoNOQP8HIS and pCcoNHisOQP were isolated and digested with *KpnI* and *SpeI* restriction enzymes. Digested samples were separated on the 1% agarose gel and 4.6 kbp his tagged fragment was inserted into 10.7 kbp vector and then transformed into One Shot TOP10 chemical competent cells. Transformants were selected on the basis of their size (15.3 kbp) and tetracycline resistance. Four transconjugants which have the desired histagtail were saved and designated as Top10/pCcoNHisOQP8HIS (Figure 3.57).

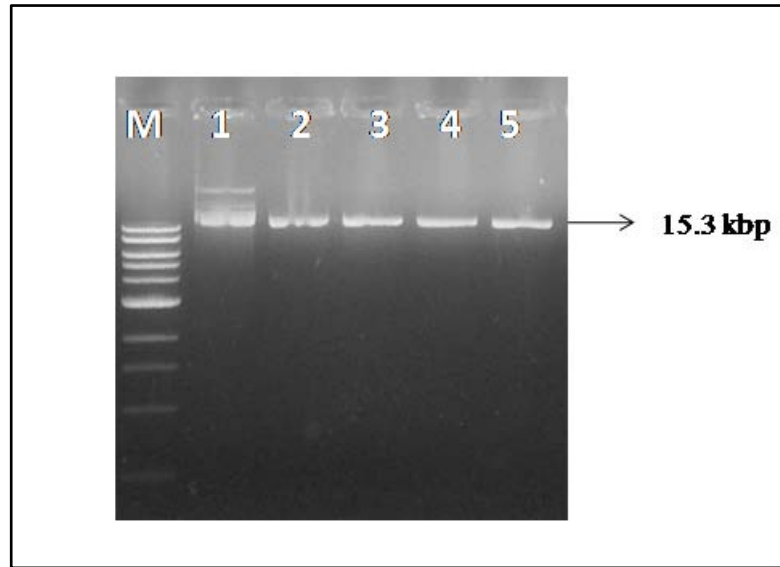


Figure 3.57 Determination of pCcoNHisOQP8HIS construct depending on size (15.3 kbp). Line 1 contains uncut pCcoNHisOQP as control; Line 2 contains, 15.3 kbp pCcoNHisOQP8HIS1 DNA; Line 3 contains, 15.3 kbp pCcoNHisOQP8HIS2 DNA; Line 4 contains, 15.3 kbp pCcoNHisOQP8HIS3 DNA; Line 5 contains, 15.3 kbp pCcoNHisOQP8HIS4 DNA.

3.7.4. NADISTaining

After transferring pCcoNHisOQP8HIS plasmid to the GK32 strain of *R. capsulatus*, the cyt *cbb*₃ oxidase activity was detected by NADI Staining. Cells grown on MedA medium were turned to deep blue after 30 secs after treatment with NADI staining. This result showed that addition of a histidine tail to the C-terminus of *ccoP* did not affect the cyt *cbb*₃ oxidase activity (Figure 3.58)

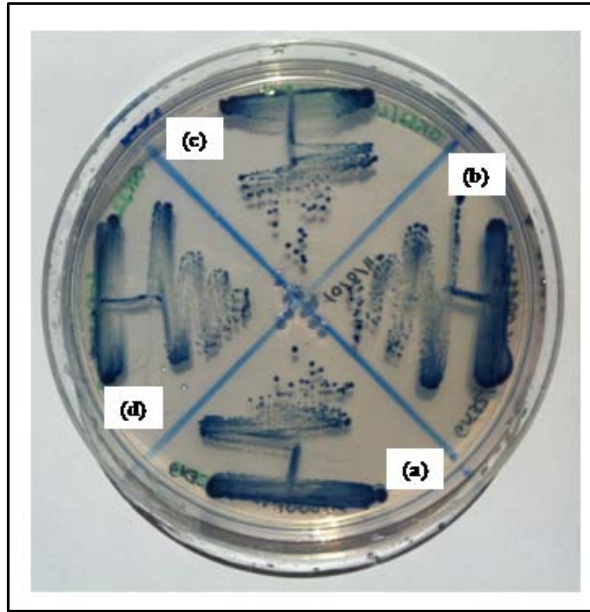


Figure 3.58 NADH staining of GK32/pCcoNHisOQP8HIS strains. **(a),(b), (c)** and **(d)** represent NADH stained *R. capsulatus* GK32/pCcoNHisOQP8HIS1, GK32/pCcoNHisOQP8HIS2, GK32/pCcoNHisOQP8HIS3 and GK32/pCcoNHisOQP8HIS4 transconjugants respectively with NADH⁺ phenotype.

Phenotypic and genetic characterization of positive and negative controls, MT1131, GK32 and GK2/pOX15 with targeted mutants, GK2/pOX15N265V, GK2/pOX15Y420F and GK2/pOX15F480A were summarized in Table 3.1

Table 3.1 Summary of the characterization experiments including, NADI staining, western blotting, SDS-PAGE/TMBZ staining, *cbb*₃-type oxidase activity and proton pumping of positive and negative controls with targeted mutants.

<i>R.capsulatus</i> strains	NADI phenotype profiles	Detection by anti-CcoN antibodies	cyt c profiles <i>C_p C₁ C_y C_o</i>	% of wild-type activity	Proton translocation H^+/e^- ratio
MT1131	+	+	+ + + +	47%	NT
GK32/pOX15	+	+	+ + + +	100%	NT
GK32	-	-	- + + -	0%	NT
GK32/pOX15N265V	-	-	- + + -	1%	NT
GK32/pOX15Y420F	-	-	- + + -	8.1/	NT
GK32/pOX15F480A	+ slow	+	+ + + +	71.8%	NT
KZ101/pOX15	+	NT	NT	NT	0.54 ±0.07 H^+/e^-
KZ101/pRK415	-	NT	NT	NT	No pumping
KZ101/pOX15F480A	+ slow	NT	NT	NT	No pumping

* For *cbb*₃-type oxidase activity, 100% activity corresponds to 1602 nmol of cyt c/mg of protein per min. (semiaerobic condition). Values shown are means of three independent measurements.

** H^+/e^- ratios are averages + standard deviation (n=10). Exogenous substrate was TMPD.

CHAPTER 4

DISCUSSION

Heme-copper oxidase superfamily is diverse in terms of their subunit numbers, present in enzymatic complex, electron donor (cytochrome c, quinol or high potential iron sulfur protein), and heme type (A,B or O), resulting in different electron and proton transfer characteristics (Pereira et al., 2001). Even they have different proton transfer characteristics, these enzymes have proton conducting channels which provides protons to the active sites of the enzymes. 72% of the HCO family includes the largest and the most studied group of the enzymes; A- family (Hemp et al., 2007). The cbb3-type cytochrome c oxidases are phylogenetically the most distant respiratory heme-copper oxidase from the aa3- type enzyme of mitochondria and bacterial cells (Pereira et al., 2001; Hemp et al., 2007). Within all the members of HCOs, only the subunit I that include O₂ reduction site is homologous. Despite the differences in conserved residues, cbb3-type oxidases have a single-proton uptake channel which is analogous to the K-channel of the aa3- type oxidases. Even sharing the similar structural features with the classical oxidases, recent studies showed that tyrosine residue in the histidine-tyrosine cross-link to the active site originates from a different helix than in the other heme-copper oxidases (Hemp et al., 2006; Rauhamäki et al., 2006; Oztürk and Watmough, 2011). Also, cbb3-type oxidases has exclusive feature compared to the other HCuo which is related with the glutamate residue that hydrogen-bonded to the proximal histidine ligand of the high-spin heme (Rauhamäki et al., 2009; Oztürk et al., 2012). In

addition to these unique properties, it has been showed that the stoichiometry of proton pumping of cbb3-type oxidase in whole cells is about 0.4 – 0.5 protons per electron which is about the half of the value obtained with the aa3- type oxygen reductases (Han et al., 2011; Murali et al., 2012). Nevertheless structural data support that the electron supply apparatus of cbb3-type oxidases completely differs from that of the other HCuOs, which presumably influences electron transfer rates and, concomitantly, catalysis and proton pumping (Buschmann et al., 2010). As it has been supported by different studies, the cbb3-type oxidases differ from all other HCuOs. In order to results of sequence analyses, structural modeling, and site-directed mutagenesis studies, conserved residues may play important role(s) in the structure and /or the function of the enzyme (Hemp et al., 2007). According to this information, mutagenesis studies on the partially conserved residues might also help to understand the minimal common properties of the superfamily.

Considering the activities of the mutants and the detection of their subunits in the membrane, results indicate that N265V mutant, in helix VI, is not assembled and has almost no activity. The position of N265 residue on the 3-D structure of cbb3-type oxidases revealed that this residue is close to one of the important histidine ligands of the active site. Thus, mutation at this position may affect the structural organization and the assembly of the enzyme.

The other mutation at the Y420 position in helix X, also caused disassembly of the enzyme however, even if it has NADI- profile, this mutant contains a functional cytochrome cbb3. Depending on this data it is suggested that Phenylalanine mutant reverts easily to Tyrosine. In site-directed mutagenesis studies tyrosine is often mutated to phenylalanine or alanine in order to study tyrosine phosphorylation. Phenylalanine is almost identical with tyrosine, except it is missing

the -OH group which is basically a “non-phosphorylatable” tyrosine. Alanine, on the other hand, totally lacks the aromatic ring (and -OH) of tyrosine, so mutating tyrosine to alanine effectively removes the tyrosine side chain from a protein. Mutation in the position of the tyrosine 420 residue in the cbb3-type oxidase from *R. capsulatus* showed that this residue has role in structure of the enzyme. However, it would be better to substitute this tyrosine residue to Methionine, Leucine or Valine in the future work.

To date, purified cbb3-type oxidases from *Bradyrhizobium japonicum* and *Helicobacter pylori* have successfully showed that they pumped protons by using the methods of reconstituted phospholipids vesicles. However, exact values of H^+/e^- ratio were not reported for *Helicobacter pylori* and for *B. japonicum*, the stoichiometry numbers varied from 0.2-0.4 (De Gier et al., 1996; Toledo-Cuevas et al., 1998). On the other hand, Pumping measurements with whole cells of *Paracoccus denitrificans* and *Rhodobacter sphaeroides* showed that the C-family oxygen reductase pumps 1 H^+/e^- (Arslan et al., 2000). The proton pumping experiments with whole cells of *R. capsulatus* complement mutant KZ101/pOX15 support the findings of Han et al., (2011) which the stoichiometry of proton pumping of cbb3-type oxidase is about 0.5 – 0.6. The mutant F480A, in helix XI, is similar to wild type oxidase in structure however it had less activity. Data showed that F480A mutation caused to loss of proton translocation ability of the enzyme. As the proton output of the HCOs is not known very well, this residue might play a role in removal of proton from the catalytic center to the P- side of the membrane.

In summary, sequence data on heme copper oxidases has allowed us to identify functionally important residues and to verify experimentally. Overall data represented in this study showed that asparagine and tyrosine residues at the position

of 265 and 420 have critical role in enzyme activity and assembly while phenylalanine residue at the position of 480 has role in the proton translocation. Further experiments including the structural determination of c-type oxygen reductases will be necessary to clarify how these enzymes function.

CHAPTER 5

FUTURE WORK

To understand the main characteristics of *cbb*₃-type oxidase enzyme, purified enzyme is required. The *cbb*₃ oxidase from *R. capsulatus* has not been purified to date, and also, the purified enzymes from different organisms were not stable and the yield was not good enough to work with. The new construct, with the histidine tail at C-terminus of the *ccoP*, created in this work is a good candidate for the purification of the *cbb*₃ oxidase from *R. capsulatus*. As future aspect, this construct will be used to purify the enzyme. The data in this thesis revealed that the proton pumping mechanism of *cbb*₃ oxidase is different from other oxidases. If the purification of the enzyme is achieved by using the new construct, it might also help for better understanding of the proton pumping mechanism of the enzyme. The isolated enzyme will also be used to understand the effects of these important partially conserved residues on the steady state reduction of oxygen. Addition to these, by using low temperature UV/visible spectroscopy the perturbations of the heme *b* and *b*₃, and by CO-recombination kinetics, changes in the kinetics of the active site due to mutations can be observed. As summary, the new construct will be used for the isolation of the *cbb*₃ oxidase from *R. capsulatus* and for many experiments which may give insight to the better understanding of the mechanism of the enzyme.

REFERENCES

- Abramson, J., S. Riistama, G. Larsson, A. Jasaitis, M. Svensson-Ek, L. Laakkonen, A. Puustinen, S. Iwata, and M. Wikström. 2000. The structure of the ubiquinol oxidase from *Escherichia coli* and its ubiquinone binding site. *Nature structural biology*. 7:910–7. doi:10.1038/82824.
- Adelroth, P., M. Ek, and P. Brzezinski. 1998. Factors determining electron-transfer rates in cytochrome *c* oxidase: investigation of the oxygen reaction in the *R. sphaeroides* enzyme. *Biochimica et biophysica acta*. 1367:107–17.
- Adelroth, P., M.S. Ek, D.M. Mitchell, R.B. Gennis, and P. Brzezinski. 1997. Glutamate 286 in cytochrome *aa*₃ from *Rhodobacter sphaeroides* is involved in proton uptake during the reaction of the fully-reduced enzyme with dioxygen. *Biochemistry*. 36:13824–9. doi:10.1021/bi9629079.
- Agmon, N. 1995. The Grotthuss mechanism. *Chemical Physics Letters*. 244:456–462. doi:10.1016/0009-2614(95)00905-J.
- Albury, M.S., C. Elliott, and A.L. Moore. 2009. Towards a structural elucidation of the alternative oxidase in plants. *Physiologia plantarum*. 137:316–27. doi:10.1111/j.1399-3054.2009.01270.x.
- Anraku, Y. 1988. Bacterial Electron Transport Chains. *Annual Review of Biochemistry*. 57:101–132. doi:10.1146/annurev.bi.57.070188.000533.
- Arslan, E., a Kannt, L. Thöny-Meyer, and H. Hennecke. 2000. The symbiotically essential *cbb*(₃)-type oxidase of *Bradyrhizobium japonicum* is a proton pump. *FEBS letters*. 470:7–10.
- Arutyunyan, A.M., V.B. Borisov, V.I. Novoderezhkin, J. Ghaim, J. Zhang, R.B. Gennis, and A.A. Konstantinov. 2008. Strong excitonic interactions in the oxygen-reducing site of *bd*-type oxidase: the Fe-to-Fe distance between hemes *d* and *b*595 is 10 Å. *Biochemistry*. 47:1752–9. doi:10.1021/bi701884g.
- Batut, J., M.L. Daveran-Mingot, M. David, J. Jacobs, A.M. Garnerone, and D. Kahn. 1989. *fixK*, a gene homologous with *fnr* and *crp* from *Escherichia coli*, regulates nitrogen fixation genes both positively and negatively in *Rhizobium meliloti*. *The EMBO journal*. 8:1279–86.
- Belevich, I. 2007. Proton Translocation Coupled to Electron Transfer Reactions in Terminal Oxidases Institute of Biotechnology.

- Belevich, I., V.B. Borisov, J. Zhang, K. Yang, A.A. Konstantinov, R.B. Gennis, and M.I. Verkhovsky. 2005. Time-resolved electrometric and optical studies on cytochrome *bd* suggest a mechanism of electron-proton coupling in the di-heme active site. *Proceedings of the National Academy of Sciences of the United States of America*. 102:3657–62. doi:10.1073/pnas.0405683102.
- Bloch, D., I. Belevich, A. Jasaitis, C. Ribacka, A. Puustinen, M.I. Verkhovsky, and M. Wikström. 2004. The catalytic cycle of cytochrome *c* oxidase is not the sum of its two halves. *Proceedings of the National Academy of Sciences of the United States of America*. 101:529–33. doi:10.1073/pnas.0306036101.
- Bogachev, A. V, R.A. Murtazina, and V.P. Skulachev. 1996. H^+/e^- Stoichiometry for NADH Dehydrogenase I and Dimethyl Sulfoxide Reductase in Anaerobically Grown *Escherichia coli* Cells. 178:6233–6237.
- Borisov, V.B., R.B. Gennis, J. Hemp, and M.I. Verkhovsky. 2011. The cytochrome *bd* respiratory oxygen reductases. *Biochimica et biophysica acta*. 1807:1398–413. doi:10.1016/j.bbabbio.2011.06.016.
- Brändén, G., R.B. Gennis, and P. Brzezinski. 2006. Transmembrane proton translocation by cytochrome *c* oxidase. *Biochimica et biophysica acta*. 1757:1052–63. doi:10.1016/j.bbabbio.2006.05.020.
- Brändén, M., F. Tomson, R.B. Gennis, and P. Brzezinski. 2002. The Entry Point of the K-Proton-Transfer Pathway in Cytochrome *c* Oxidase. *Biochemistry*. 41:10794–10798. doi:10.1021/bi026093+.
- Brenner, D.J., N.R. Krieg, and J. Staley. 2005. Bergey's manual of systematic bacteriology Vol. 2, The proteobacteria. Part C, The Alpha-, Beta-, Delta-, and Epsilonproteobacteria. 2nd ed. Springer, New York.
- Brochier-Armanet, C., E. Talla, and S. Gribaldo. 2009. The multiple evolutionary histories of dioxygen reductases: Implications for the origin and evolution of aerobic respiration. *Molecular biology and evolution*. 26:285–97. doi:10.1093/molbev/msn246.
- Buschmann, S., E. Warkentin, H. Xie, J.D. Langer, U. Ermler, and H. Michel. 2010. The structure of *cbb*₃ cytochrome oxidase provides insights into proton pumping. *Science (New York, N.Y.)*. 329:327–30. doi:10.1126/science.1187303.
- Calhoun, M.W., J.W. Thomas, and R.B. Gennis. 1994. The cytochrome oxidase superfamily of redox-driven proton pumps. *Trends in biochemical sciences*. 19:325–30.
- Carroll, J., I.M. Fearnley, J.M. Skehel, R.J. Shannon, J. Hirst, and J.E. Walker. 2006. Bovine complex I is a complex of 45 different subunits. *The Journal of biological chemistry*. 281:32724–7. doi:10.1074/jbc.M607135200.

- Castresana, J., M. Lübben, and M. Saraste. 1995. New archaeobacterial genes coding for redox proteins: implications for the evolution of aerobic metabolism. *Journal of molecular biology*. 250:202–10. doi:10.1006/jmbi.1995.0371.
- Castresana, J., and M. Saraste. 1995. Evolution of energetic metabolism: the respiration-early hypothesis. *Trends in biochemical sciences*. 20:443–8.
- Castresana, J., M. Lubben, M. Saraste, and D.G. Higgins. 1994. Evolution of cytochrome oxidase, an enzyme older than atmospheric oxygen. *EMBO Journal*. 13:2516–2525.
- Chang, H.-Y., J. Hemp, Y. Chen, J. a Fee, and R.B. Gennis. 2009. The cytochrome *ba*₃ oxygen reductase from *Thermus thermophilus* uses a single input channel for proton delivery to the active site and for proton pumping. *Proceedings of the National Academy of Sciences of the United States of America*. 106:16169–73. doi:10.1073/pnas.0905264106.
- Cline, J., J.C. Braman, and H.H. Hogrefe. 1996. PCR fidelity of *pfu* DNA polymerase and other thermostable DNA polymerases. *Nucleic acids research*. 24:3546–51.
- Cobine, P.A., F. Pierrel, and D.R. Winge. 2006. Copper trafficking to the mitochondrion and assembly of copper metalloenzymes. *Biochimica et biophysica acta*. 1763:759–72. doi:10.1016/j.bbamcr.2006.03.002.
- Crofts, A.R. 2004. The Q-cycle - A Personal Perspective. *Photosynthesis research*. 80:223–43. doi:10.1023/B:PRES.0000030444.52579.10.
- Cukierman, S. 2006. Et tu, Grotthuss! and other unfinished stories. *Biochimica et biophysica acta*. 1757:876–85. doi:10.1016/j.bbabbio.2005.12.001.
- Daldal, F. 1988. Cytochrome *c*₂-independent respiratory growth of *Rhodobacter capsulatus*. *Journal of bacteriology*. 170:2388–91.
- Daldal, F., S. Cheng, J. Applebaum, E. Davidson, and R.C. Prince. 1986. Cytochrome *c*₂ is not essential for photosynthetic growth of *Rhodospseudomonas capsulata*. *Proceedings of the National Academy of Sciences of the United States of America*. 83:2012–6.
- Davis, J., T.J. Donohue, and S. Kaplan. 1988. Construction, characterization, and complementation of a Puf⁻ mutant of *Rhodobacter sphaeroides*. *Journal of bacteriology*. 170:320–9.
- Ditta, G., T. Schmidhauser, E. Yakobson, P. Lu, X.W. Liang, D.R. Finlay, D. Guiney, and D.R. Helinski. 1985. Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. *Plasmid*. 13:149–53.

- Ducluzeau, A.-L., S. Ouchane, and W. Nitschke. 2008. The *cbb₃* oxidases are an ancient innovation of the domain bacteria. *Molecular biology and evolution*. 25:1158–66. doi:10.1093/molbev/msn062.
- Efremov, R.G., R. Baradaran, and L.A. Sazanov. 2010. The architecture of respiratory complex I. *Nature*. 465:441–5. doi:10.1038/nature09066.
- Ekici, S., G. Pawlik, E. Lohmeyer, H.-G. Koch, and F. Daldal. 2012. Biogenesis of *cbb₃*-type cytochrome *c* oxidase in *Rhodobacter capsulatus*. *Biochimica et biophysica acta*. 1817:898–910. doi:10.1016/j.bbabi.2011.10.011.
- Fetter, J.R., J. Qian, J. Shapleigh, J.W. Thomas, A. García-Horsman, E. Schmidt, J. Hosler, G.T. Babcock, R.B. Gennis, and S. Ferguson-Miller. 1995. Possible proton relay pathways in cytochrome *c* oxidase. *Proceedings of the National Academy of Sciences of the United States of America*. 92:1604–8.
- Flaman, J.M., T. Frebourg, V. Moreau, F. Charbonnier, C. Martin, C. Ishioka, S.H. Friend, and R. Iggo. 1994. A rapid PCR fidelity assay. *Nucleic acids research*. 22:3259–60.
- Flock, U., J. Reimann, and P. Adelroth. 2006. Proton transfer in bacterial nitric oxide reductase. *Biochemical Society transactions*. 34:188–90. doi:10.1042/BST0340188.
- Florens, L., B. Schmidt, J. McCracken, and S. Ferguson-Miller. 2001. Fast Deuterium Access to the Buried Magnesium/Manganese Site in Cytochrome *c* Oxidase. *Biochemistry*. 40:7491–7497. doi:10.1021/bi0101188.
- Forte, E., a Urbani, M. Saraste, P. Sarti, M. Brunori, and a Giuffrè. 2001. The cytochrome *cbb₃* from *Pseudomonas stutzeri* displays nitric oxide reductase activity. *European journal of biochemistry / FEBS*. 268:6486–91.
- Fuller, S.D., V.M. Darley-USmar, and R.A. Capaldi. 1981. Covalent complex between yeast cytochrome *c* and beef heart cytochrome *c* oxidase which is active in electron transfer. *Biochemistry*. 20:7046–53.
- Garcia-Horsman, J.A., B. Barquera, J. Rumbley, J. Ma, and R.B. Gennis. 1994a. MINIREVIEW The Superfamily of Heme-Copper Respiratory Oxidases. *Journal of bacteriology*. 176:5587–5600.
- Garcia-Horsman, J.A., E. Berry, J.P. Shapleigh, J.O. Alben, and R.B. Gennis. 1994b. A Novel Cytochrome *c* Oxidase from *Rhodobacter sphaeroides* That Lacks CuA. *Biochemistry*. 33:3113–3119. doi:10.1021/bi00176a046.
- Garcia-Horsman, J.A., A. Puustinen, R.B. Gennis, and M. Wikström. 1995. Proton transfer in cytochrome *bo₃* ubiquinol oxidase of *Escherichia coli*: second-site mutations in subunit I that restore proton pumping in the mutant Asp135-->Asn. *Biochemistry*. 34:4428–33.

- De Gier, J.W., M. Schepper, W.N. Reijnders, S.J. van Dyck, D.J. Slotboom, A. Warne, M. Saraste, K. Krab, M. Finel, A.H. Stouthamer, R.J. van Spanning, and J. van der Oost. 1996. Structural and functional analysis of *aa*₃-type and *cbb*₃-type cytochrome *c* oxidases of *Paracoccus denitrificans* reveals significant differences in proton-pump design. *Molecular microbiology*. 20:1247–60.
- Gray, K. a, M. Grooms, H. Myllykallio, C. Moomaw, C. Slaughter, and F. Daldal. 1994. *Rhodobacter capsulatus* contains a novel *cb*-type cytochrome *c* oxidase without a Cu_A center. *Biochemistry*. 33:3120–7.
- Gribaldo, S., E. Talla, and C. Brochier-Armanet. 2009. Evolution of the haem copper oxidases superfamily: a rooting tale. *Trends in biochemical sciences*. 34:375–81. doi:10.1016/j.tibs.2009.04.002.
- De Grotthuss, C.J.T. 1806. Sur la décomposition de l'eau et des corps qu'elle tient en dissolution à l'aide de l'électricité galvanique. *Annali di Chimica*. 58:54–73.
- Han, H., J. Hemp, L.A. Pace, H. Ouyang, K. Ganesan, J.H. Roh, F. Daldal, S.R. Blanke, and R.B. Gennis. 2011. Adaptation of aerobic respiration to low O₂ environments. *Proceedings of the National Academy of Sciences of the United States of America*. 108:14109–14. doi:10.1073/pnas.1018958108.
- Hemp, J., C. Christian, B. Barquera, R.B. Gennis, and T.J. Martínez. 2005. Helix switching of a key active-site residue in the cytochrome *cbb*₃ oxidases. *Biochemistry*. 44:10766–75. doi:10.1021/bi050464f.
- Hemp, J., and R.B. Gennis. 2008. Diversity of the heme-copper superfamily in archaea: insights from genomics and structural modeling. *Results and problems in cell differentiation*. 45:1–31. doi:10.1007/400_2007_046.
- Hemp, J., H. Han, J.H. Roh, S. Kaplan, T.J. Martinez, and R.B. Gennis. 2007. Comparative genomics and site-directed mutagenesis support the existence of only one input channel for protons in the C-family (*cbb*₃ oxidase) of heme-copper oxygen reductases. *Biochemistry*. 46:9963–72. doi:10.1021/bi700659y.
- Hemp, J., D.E. Robinson, K.B. Ganesan, T.J. Martinez, N.L. Kelleher, and R.B. Gennis. 2006. Evolutionary migration of a post-translationally modified active-site residue in the proton-pumping heme-copper oxygen reductases. *Biochemistry*. 45:15405–10. doi:10.1021/bi062026u.
- Hemp, J., D.E. Robinson, T.J. Martinez, N.L. Kelleher, and B. Robert. 2008. The Evolutionary Migration of a Post-Translationally Modified Active-Site Residue in the Proton-Pumping Heme-Copper Oxygen Reductases. *Biochemistry*. 45:15405–15410. doi:10.1021/bi062026u..
- Hendriks, J.H.M., A. Jasaitis, M. Saraste, and M.I. Verkhovsky. 2002. Proton and electron pathways in the bacterial nitric oxide reductase. *Biochemistry*. 41:2331–40.

- Hill, B.C., and C. Greenwood. 1984. Kinetic evidence for the re-definition of electron transfer pathways from cytochrome *c* to O₂ within cytochrome oxidase. *FEBS letters*. 166:362–6.
- Hill, J.J., J.O. Alben, and R.B. Gennis. 1993. Spectroscopic evidence for a heme-heme binuclear center in the cytochrome *bd* ubiquinol oxidase from *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America*. 90:5863–7.
- Hino, T., Y. Matsumoto, S. Nagano, H. Sugimoto, Y. Fukumori, T. Murata, S. Iwata, and Y. Shiro. 2010. Structural basis of biological N₂O generation by bacterial nitric oxide reductase. *Science (New York, N.Y.)*. 330:1666–70. doi:10.1126/science.1195591.
- Hochkoeppler, A., F.E. Jenney, S.E. Lang, D. Zannoni, and F. Daldal. 1995. Membrane-associated cytochrome *c_y* of *Rhodobacter capsulatus* is an electron carrier from the cytochrome *bc₁* complex to the cytochrome *c* oxidase during respiration. *Journal of bacteriology*. 177:608–13.
- Hosler, J.P., S. Ferguson-Miller, M.W. Calhoun, J.W. Thomas, J. Hill, L. Lemieux, J. Ma, C. Georgiou, J. Fetter, J. Shapleigh, M.M.J. Tecklenburg, G.T. Babcock, and R.B. Gennis. 1993. Insight into the active-site structure and function of cytochrome oxidase by analysis of site-directed mutants of bacterial cytochrome *aa₃* and cytochrome *bo*. *Journal of Bioenergetics and Biomembranes*. 25:121–36. doi:10.1007/BF00762854.
- Huang, L., G. Sun, D. Cobessi, A.C. Wang, J.T. Shen, E.Y. Tung, V.E. Anderson, and E.A. Berry. 2006. 3-nitropropionic acid is a suicide inhibitor of mitochondrial respiration that, upon oxidation by complex II, forms a covalent adduct with a catalytic base arginine in the active site of the enzyme. *The Journal of biological chemistry*. 281:5965–72. doi:10.1074/jbc.M511270200.
- Humphrey, W., A. Dalke, and K. Schulten. 1996. VMD: visual molecular dynamics. *Journal of Molecular Graphics*. 14:33–38. doi:10.1016/0263-7855(96)00018-5.
- Imhoff, F.J. 1995. Anoxygenic Photosynthetic Bacteria. In *Taxonomy and physiology of phototrophic purple bacteria and green sulfur bacteria*. R.E. Blankenship, M.T. Madigan, and C.E. Bauer, editors. Kluwer Academic Publishers, Dordrecht, The Netherlands. 1–15.
- Iwata, S., J.K.J.W.K. Lee, K. Okada, M. Iwata, B. Rasmussen, T.A. Link, S. Ramaswamy, and B.K. Jap. 1998. Complete Structure of the 11-Subunit Bovine Mitochondrial Cytochrome *bc₁* Complex. *Science*. 281:64–71. doi:10.1126/science.281.5373.64.
- Iwata, S., C. Ostermeier, B. Ludwig, and H. Michel. 1995. Structure of 28 Å resolution of cytochrome *c* oxidase. *Nature*. 376:660–69.

- Jenney, F.E., and F. Daldal. 1993. A novel membrane-associated *c*-type cytochrome, cyt *c_y*, can mediate the photosynthetic growth of *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*. *The EMBO journal*. 12:1283–92.
- Jenney, F.E., R.C. Prince, and F. Daldal. 1994. Roles of the Soluble Cytochrome *c₂* and Membrane-Associated Cytochrome *c_y* of *Rhodobacter capsulatus* in Photosynthetic Electron Transfer. *Biochemistry*. 33:2496–2502. doi:10.1021/bi00175a019.
- Kaila, V.R.I., M.P. Johansson, D. Sundholm, L. Laakkonen, and M. Wiström. 2009. The chemistry of the Cu_B site in cytochrome *c* oxidase and the importance of its unique His-Tyr bond. *Biochimica et biophysica acta*. 1787:221–33.
- Keilin, D. 1966. The history of cell respiration and cytochrome. Cambridge University Press, Cambridge, United Kingdom.
- Kobayashi, K., H. Une, and K. Hayashi. 1989. Electron transfer process in cytochrome oxidase after pulse radiolysis. *The Journal of biological chemistry*. 264:7976–80.
- Koch, H.G., O. Hwang, and F. Daldal. 1998. Isolation and characterization of *Rhodobacter capsulatus* mutants affected in cytochrome *cbb₃* oxidase activity. *Journal of bacteriology*. 180:969–78.
- Koch, H.G., C. Winterstein, A.S. Saribas, J.O. Alben, and F. Daldal. 2000. Roles of the *ccoGHIS* gene products in the biogenesis of the *cbb₃*-type cytochrome *c* oxidase. *Journal of molecular biology*. 297:49–65. doi:10.1006/jmbi.2000.3555.
- Kompantseva, E.J. 1981. Utilization of sulfide by nonsulfur purple bacteria *Rhodopseudomonas capsulata*. *Mikrobiologiya*. 50:429–436.
- Konstantinov, A.A., S. Siletsky, D. Mitchell, A. Kaulen, and R.B. Gennis. 1997. The roles of the two proton input channels in cytochrome *c* oxidase from *Rhodobacter sphaeroides* probed by the effects of site-directed mutations on time-resolved electrogenic intraprotein proton transfer. *Proceedings of the National Academy of Sciences of the United States of America*. 94:9085–90.
- Kulajta, C., J.O. Thumfart, S. Haid, F. Daldal, and H.-G. Koch. 2006. Multi-step assembly pathway of the *cbb₃*-type cytochrome *c* oxidase complex. *Journal of molecular biology*. 355:989–1004. doi:10.1016/j.jmb.2005.11.039.
- Lacks, S., and B. Greenberg. 1977. Complementary specificity of restriction endonucleases of *Diplococcus pneumoniae* with respect to DNA methylation. *Journal of molecular biology*. 114:153–68.
- Laemmli, U.K. 1970. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature*. 227:680–685. doi:10.1038/227680a0.

- Lee, H.J., R.B. Gennis, and P. Ådelroth. 2011. Entrance of the proton pathway in *cbb₃*-type heme-copper oxidases. *Proceedings of the National Academy of Sciences of the United States of America*. 108:17661–6. doi:10.1073/pnas.1107543108.
- Lee, H.J., J. Reimann, Y. Huang, and P. Adelroth. 2012. Functional proton transfer pathways in the heme-copper oxidase superfamily. *Biochimica et biophysica acta*. 1817:537–44. doi:10.1016/j.bbabbio.2011.10.007.
- Lee, H.M., T.K. Das, D.L. Rousseau, D. Mills, S. Ferguson-Miller, and R.B. Gennis. 2000. Mutations in the putative H-channel in the cytochrome *c* oxidase from *Rhodobacter sphaeroides* show that this channel is not important for proton conduction but reveal modulation of the properties of heme *a*. *Biochemistry*. 39:2989–96.
- Lemon, D.D., M.W. Calhoun, R.B. Gennis, and W.H. Woodruff. 1993. The gateway to the active site of heme-copper oxidases. *Biochemistry*. 32:11953–11956. doi:10.1021/bi00096a002.
- Li, Y., A. Hopper, T. Overton, D.J.P. Squire, J. Cole, and N. Tovell. 2010. Organization of the electron transfer chain to oxygen in the obligate human pathogen *Neisseria gonorrhoeae*: roles for cytochromes *c₄* and *c₅*, but not cytochrome *c₂*, in oxygen reduction. *Journal of bacteriology*. 192:2395–406. doi:10.1128/JB.00002-10.
- Ma, J., P.H. Tsatsos, D. Zaslavsky, B. Barquera, J.W. Thomas, A. Katsonouri, A. Puustinen, M. Wikström, P. Brzezinski, J.O. Alben, and R.B. Gennis. 1999. Glutamate-89 in subunit II of cytochrome *bo₃* from *Escherichia coli* is required for the function of the heme-copper oxidase. *Biochemistry*. 38:15150–6.
- Madigan, M.T. 1988. Microbiology, physiology, and ecology of phototrophic bacteria. In A.J.B. Zehnder (ed) *Biology of Anaerobic Microorganisms*. John Wiley & Sons, New York. 39–111.
- Madigan, M.T., and H. Gest. 1979. Growth of the photosynthetic bacterium *Rhodopseudomonas capsulata* chemoautotrophically in darkness with H₂ as the energy source. *Journal of bacteriology*. 137:524–30.
- Marcus, R., and N. Sutin. 1985. Electron transfers in chemistry and biology. *Biochimica et biophysica acta*. 265–332.
- Marrs, B., and H. Gest. 1973. Genetic mutations affecting the respiratory electron-transport system of the photosynthetic bacterium *Rhodopseudomonas capsulata*. *Journal of bacteriology*. 114:1045–51.
- Meyer, T.E., and T.J. Donohue. 1995. Cytochromes, iron-sulfur, and copper proteins mediating electron transfer from the cyt *bc₁* complex to photosynthetic reaction center complexes. In *Anoxygenic photosynthetic bacteria*. R.E. Blankenship, M.T.

- Madigan, and C.E. Bauer, editors. Kluwer Academic Publishers, Dordrecht, The Netherlands. 725.
- Miller, M.J., and R.B. Gennis. 1985. The cytochrome *bd* complex is a coupling site in the aerobic respiratory chain of *Escherichia coli*. *The Journal of biological chemistry*. 260:14003–8.
- Mirkin, N., J. Jaconcic, V. Stojanoff, and A. Moreno. 2008. High resolution X-ray crystallographic structure of bovine heart cytochrome *c* and its application to the design of an electron transfer biosensor. *Proteins*. 70:83–92. doi:10.1002/prot.21452.
- Mitchell, P. 1961. Coupling of Phosphorylation to Electron and Hydrogen Transfer by a Chemi-Osmotic type of Mechanism. *Nature*. 191:144–148. doi:10.1038/191144a0.
- Mitchell, P. 1976. Possible molecular mechanisms of the protonmotive function of cytochrome systems. *Journal of Theoretical Biology*. 62:327–367. doi:10.1016/0022-5193(76)90124-7.
- Mosley, C.S., J.Y. Suzuki, and C.E. Bauer. 1994. Identification and molecular genetic characterization of a sensor kinase responsible for coordinately regulating light harvesting and reaction center gene expression in response to anaerobiosis. *Journal of bacteriology*. 176:7566–73.
- Murali, R., G.G. Yildiz, F. Daldal, and R.B. Gennis. 2012. Stoichiometry of proton pumping by the *cbb₃*-type oxygen reductase in whole cells of *Rhodobacter capsulatus* at pH 7 is about 0.5 H⁺ per electron. *Proceedings of the National Academy of Sciences of the United States of America*. 109:E2144; author reply E2145. doi:10.1073/pnas.1207216109.
- Myllykallio, H., and U. Liebl. 2000. Dual role for cytochrome *cbb₃* oxidase in clinically relevant proteobacteria? *Trends in microbiology*. 8:542–3.
- Oh, J.I., and S. Kaplan. 1999. The *cbb₃* terminal oxidase of *Rhodobacter sphaeroides* 2.4.1: structural and functional implications for the regulation of spectral complex formation. *Biochemistry*. 38:2688–96. doi:10.1021/bi9825100.
- Oliveberg, M., P. Brzezinski, and B.G. Malmström. 1989. The effect of pH and temperature on the reaction of fully reduced and mixed-valence cytochrome *c* oxidase with dioxygen. *Biochimica et biophysica acta*. 977:322–8.
- Ozturk, M., E. Gurel, N.J. Watmough, and S. Mandaci. 2007. Site-directed mutagenesis of five conserved residues of subunit I of the cytochrome *cbb₃* oxidase in *Rhodobacter capsulatus*. *Journal of Biochemistry and Molecular Biology*. 40:697–707.

- Oztürk, M., Ş. Er, and G.G. Yıldız. 2012. Replacement of the Glu380 with Gln380 in subunit I of cytochrome *cbb*₃ oxidase from *Rhodobacter capsulatus* results in inactive enzyme. 37:48–53. doi:10.5505/t.
- Oztürk, M., and S. Mandaci. 2007. Two conserved non-canonical histidines are essential for activity of the *cbb*(₃)-type oxidase in *Rhodobacter capsulatus*: non-canonical histidines are essential for *cbb*(₃)-type oxidase activity in *R. capsulatus*. *Molecular biology reports*. 34:165–72. doi:10.1007/s11033-006-9031-9.
- Oztürk, M., and N.J. Watmough. 2011. Mutagenesis of tyrosine residues within helix VII in subunit I of the cytochrome *cbb*₃ oxidase from *Rhodobacter capsulatus*. *Molecular biology reports*. 38:3319–26. doi:10.1007/s11033-010-0437-z.
- Parkhill, J., B.W. Wren, K. Mungall, J.M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R.M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A. V Karlyshev, S. Moule, M.J. Pallen, C.W. Penn, M.A. Quail, M.A. Rajandream, K.M. Rutherford, A.H. van Vliet, S. Whitehead, and B.G. Barrell. 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature*. 403:665–8. doi:10.1038/35001088.
- Pawlik, G., C. Kulajta, I. Sachelaru, S. Schröder, B. Waidner, P. Hellwig, F. Daldal, and H.-G. Koch. 2010. The putative assembly factor CcoH is stably associated with the *cbb*₃-type cytochrome oxidase. *Journal of bacteriology*. 192:6378–89. doi:10.1128/JB.00988-10.
- Pelletier, H., and J. Kraut. 1992. Crystal structure of a complex between electron transfer partners, cytochrome *c* peroxidase and cytochrome *c*. *Science (New York, N.Y.)*. 258:1748–55.
- Pereira, M.M., M. Santana, C.M. Soares, J. Mendes, J.N. Carita, A.S. Fernandes, M. Saraste, M.A. Carrondo, and M. Teixeira. 1999. The *caa*₃ terminal oxidase of the thermohalophilic bacterium *Rhodothermus marinus*: a HiPIP:oxygen oxidoreductase lacking the key glutamate of the D-channel. *Biochimica et biophysica acta*. 1413:1–13.
- Pereira, M.M., M. Santana, and M. Teixeira. 2001. A novel scenario for the evolution of haem-copper oxygen reductases. *Biochimica et biophysica acta*. 1505:185–208.
- Pereira, M.M., M.L. Verkhovskaya, M. Teixeira, and M.I. Verkhovsky. 2000. The *caa*(₃) terminal oxidase of *Rhodothermus marinus* lacking the key glutamate of the D-channel is a proton pump. *Biochemistry*. 39:6336–40.
- Peters, A., C. Kulajta, G. Pawlik, F. Daldal, H.-G. Koch, and C. Interactions. 2008. Stability of the *cbb*₃-type cytochrome oxidase requires specific CcoQ-CcoP interactions. *Journal of bacteriology*. 190:5576–86. doi:10.1128/JB.00534-08.
- Pfützner, U., A. Odenwald, T. Ostermann, L. Weingard, B. Ludwig, and O.M. Richter. 1998. Cytochrome *c* oxidase (heme *aa*₃) from *Paracoccus denitrificans*:

- analysis of mutations in putative proton channels of subunit I. *Journal of bioenergetics and biomembranes*. 30:89–97.
- Philippot, L. 2005. Denitrification in pathogenic bacteria: for better or worst? *Trends in microbiology*. 13:191–2. doi:10.1016/j.tim.2005.03.001.
- Pitcher, R.S., M.R. Cheesman, and N.J. Watmough. 2002. Molecular and spectroscopic analysis of the cytochrome *cbb*₍₃₎ oxidase from *Pseudomonas stutzeri*. *The Journal of biological chemistry*. 277:31474–83. doi:10.1074/jbc.M204103200.
- Pitcher, R.S., and N.J. Watmough. 2004. The bacterial cytochrome *cbb*₃ oxidases. *Biochimica et biophysica acta*. 1655:388–99. doi:10.1016/j.bbabi.2003.09.017.
- Preisig, O., D. Anthamatten, and H. Hennecke. 1993. Genes for a microaerobically induced oxidase complex in *Bradyrhizobium japonicum* are essential for a nitrogen-fixing endosymbiosis. *Proceedings of the National Academy of Sciences of the United States of America*. 90:3309–13.
- Preisig, O., R. Zufferey, and H. Hennecke. 1996a. The *Bradyrhizobium japonicum* *fixGHIS* genes are required for the formation of the high-affinity *cbb*₃-type cytochrome oxidase. *Archives of microbiology*. 165:297–305.
- Preisig, O., R. Zufferey, L. Thöny-Meyer, C. Appleby, and H. Hennecke. 1996b. A high-affinity *cbb*₃-type cytochrome oxidase terminates the symbiosis-specific respiratory chain of *Bradyrhizobium japonicum*. *Journal of bacteriology*. 178:1532–8.
- Proshlyakov, D.A., M.A. Pressler, C. DeMaso, J.F. Leykam, D.L. DeWitt, and G.T. Babcock. 2000. Oxygen activation and reduction in respiration: involvement of redox-active tyrosine 244. *Science (New York, N.Y.)*. 290:1588–91.
- Qin, L., C. Hiser, A. Mulichak, R.M. Garavito, and S. Ferguson-Miller. 2006. Identification of conserved lipid/detergent-binding sites in a high-resolution structure of the membrane protein cytochrome *c* oxidase. *Proceedings of the National Academy of Sciences of the United States of America*. 103:16117–22. doi:10.1073/pnas.0606149103.
- Rauhamäki, V., M. Baumann, R. Soliymani, A. Puustinen, and M. Wikström. 2006. Identification of a histidine-tyrosine cross-link in the active site of the *cbb*₃-type cytochrome *c* oxidase from *Rhodobacter sphaeroides*. *Proceedings of the National Academy of Sciences of the United States of America*. 103:16135–40. doi:10.1073/pnas.0606254103.
- Rauhamäki, V., D. Bloch, M.I. Verkhovsky, and M. Wikström. 2009. Active site of cytochrome *cbb*₃. *The Journal of biological chemistry*. 284:11301–8. doi:10.1074/jbc.M808839200.

- Reimann, J., U. Flock, H. Lepp, A. Honigsmann, and P. Adelroth. 2007. A pathway for protons in nitric oxide reductase from *Paracoccus denitrificans*. *Biochimica et biophysica acta*. 1767:362–73. doi:10.1016/j.bbabo.2007.03.006.
- Rey, F.E., and C.S. Harwood. 2010. *FixK*, a global regulator of microaerobic growth, controls photosynthesis in *Rhodospseudomonas palustris*. *Molecular microbiology*. 75:1007–20. doi:10.1111/j.1365-2958.2009.07037.x.
- Ribacka, C., M.I. Verkhovsky, I. Belevich, D.A. Bloch, A. Puustinen, and M. Wikström. 2005. An elementary reaction step of the proton pump is revealed by mutation of tryptophan-164 to phenylalanine in cytochrome *c* oxidase from *Paracoccus denitrificans*. *Biochemistry*. 44:16502–12. doi:10.1021/bi0511336.
- Riistama, S., A. Puustinen, A. García-Horsman, S. Iwata, H. Michel, and M. Wikström. 1996. Channelling of dioxygen into the respiratory enzyme. *Biochimica et biophysica acta*. 1275:1–4.
- Riistama, S., M.I. Verkhovsky, L. Laakkonen, M. Wikström, and A. Puustinen. 2000. Interaction between the formyl group of heme *a* and arginine 54 in cytochrome *aa₃* from *Paracoccus denitrificans*. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*. 1456:1–4. doi:10.1016/S0005-2728(99)00097-3.
- Salomonsson, L., A. Lee, R.B. Gennis, and P. Brzezinski. 2004. A single-amino-acid lid renders a gas-tight compartment within a membrane-bound transporter. *Proceedings of the National Academy of Sciences of the United States of America*. 101:11617–21. doi:10.1073/pnas.0402242101.
- Saraste, M. 1990. Structural features of cytochrome oxidase. *Quarterly reviews of biophysics*. 23:331–66.
- Saraste, M., and J. Castresana. 1994. Cytochrome oxidase evolved by tinkering with denitrification enzymes. *FEBS letters*. 341:1–4.
- Sazanov, L.A., and P. Hinchliffe. 2006. Structure of the hydrophilic domain of respiratory complex I from *Thermus thermophilus*. *Science (New York, N.Y.)*. 311:1430–6. doi:10.1126/science.1123809.
- Schägger, H., and G. von Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical biochemistry*. 166:368–79.
- Schmidt, B., J. McCracken, and S. Ferguson-Miller. 2003. A discrete water exit pathway in the membrane protein cytochrome *c* oxidase. *Proceedings of the National Academy of Sciences of the United States of America*. 100:15539–42. doi:10.1073/pnas.2633243100.
- Sganga, M.W., and C.E. Bauer. 1992. Regulatory factors controlling photosynthetic reaction center and light-harvesting gene expression in *Rhodobacter capsulatus*. *Cell*. 68:945–54.

- Shinzawa-Itoh, K., H. Aoyama, K. Muramoto, H. Terada, T. Kurauchi, Y. Tadehara, A. Yamasaki, T. Sugimura, S. Kurono, K. Tsujimoto, T. Mizushima, E. Yamashita, T. Tsukihara, and S. Yoshikawa. 2007. Structures and physiological roles of 13 integral lipids of bovine heart cytochrome *c* oxidase. *The EMBO journal*. 26:1713–25. doi:10.1038/sj.emboj.7601618.
- Siletsky, S.A., I. Belevich, A. Jasaitis, A.A. Konstantinov, M. Wikström, T. Soulimane, and M.I. Verkhovsky. 2007. Time-resolved single-turnover of *ba*₃ oxidase from *Thermus thermophilus*. *Biochimica et biophysica acta*. 1767:1383–92. doi:10.1016/j.bbabbio.2007.09.010.
- Sistrom, W.R. 1960. A Requirement for Sodium in the Growth of *Rhodopseudomonas spheroides*. *Microbiology*. 22:778–785. doi:10.1099/00221287-22-3-778.
- Soulimane, T., G. Buse, G.P. Bourenkov, H.D. Bartunik, R. Huber, and M.E. Than. 2000. Structure and mechanism of the aberrant *ba*(₃)-cytochrome *c* oxidase from *Thermus thermophilus*. *The EMBO journal*. 19:1766–76. doi:10.1093/emboj/19.8.1766.
- Sousa, F.L., R.J. Alves, J.B. Pereira-Leal, M. Teixeira, and M.M. Pereira. 2011. A bioinformatics classifier and database for heme-copper oxygen reductases. *PloS one*. 6:e19117. doi:10.1371/journal.pone.0019117.
- Sousa, F.L., R.J. Alves, M.A. Ribeiro, J.B. Pereira-Leal, M. Teixeira, and M.M. Pereira. 2012. The superfamily of heme-copper oxygen reductases: types and evolutionary considerations. *Biochimica et biophysica acta*. 1817:629–37. doi:10.1016/j.bbabbio.2011.09.020.
- Stock, D., A.G. Leslie, and J.E. Walker. 1999. Molecular architecture of the rotary motor in ATP synthase. *Science (New York, N.Y.)*. 286:1700–5.
- Stoscheck, C.M. 1990. Quantitation of protein. *Methods in enzymology*. 182:50–68.
- Svensson-Ek, M., J. Abramson, G. Larsson, S. Törnroth, P. Brzezinski, and S. Iwata. 2002. The X-ray crystal structures of wild-type and EQ(I-286) mutant cytochrome *c* oxidases from *Rhodobacter sphaeroides*. *Journal of molecular biology*. 321:329–39.
- Swem, D.L., and C.E. Bauer. 2002. Coordination of Ubiquinol Oxidase and Cytochrome *cbb*₃ Oxidase Expression by Multiple Regulators in *Rhodobacter capsulatus*. *Journal of bacteriology*. 184:2815–2820. doi:10.1128/JB.184.10.2815.
- Swem, L.R., S. Elsen, T.H. Bird, D.L. Swem, H.G. Koch, H. Myllykallio, F. Daldal, and C.E. Bauer. 2001. The RegB/RegA two-component regulatory system controls synthesis of photosynthesis and respiratory electron transfer components in *Rhodobacter capsulatus*. *Journal of molecular biology*. 309:121–38. doi:10.1006/jmbi.2001.4652.

- Szundi, I., C. Funatogawa, J.A. Fee, T. Soulimane, and O. Einarsdóttir. 2010. CO impedes superfast O₂ binding in *ba*₃ cytochrome oxidase from *Thermus thermophilus*. *Proceedings of the National Academy of Sciences of the United States of America*. 107:21010–5. doi:10.1073/pnas.1008603107.
- Thomas, J.W., A. Puustinen, J.O. Alben, R.B. Gennis, and M. Wikstrom. 1993. Substitution of asparagine for aspartate-135 in subunit I of the cytochrome *bo* ubiquinol oxidase of *Escherichia coli* eliminates proton-pumping activity. *Biochemistry*. 32:10923–10928. doi:10.1021/bi00091a048.
- Thomas, P.E., D. Ryan, and W. Levin. 1976. An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate polyacrylamide gels. *Analytical biochemistry*. 75:168–76.
- Thöny-Meyer, L., C. Beck, O. Preisig, and H. Hennecke. 1994. The *ccoNOQP* gene cluster codes for a *cb*-type cytochrome oxidase that functions in aerobic respiration of *Rhodobacter capsulatus*. *Molecular microbiology*. 14:705–16.
- Toledo-Cuevas, M., B. Barquera, R.B. Gennis, M. Wikström, and J.A. García-Horsman. 1998. The *cbb*₃-type cytochrome *c* oxidase from *Rhodobacter sphaeroides*, a proton-pumping heme-copper oxidase. *Biochimica et biophysica acta*. 1365:421–34.
- Tomb, J.F., O. White, A.R. Kerlavage, R.A. Clayton, G.G. Sutton, R.D. Fleischmann, K.A. Ketchum, H.P. Klenk, S. Gill, B.A. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E.F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H.G. Khalak, A. Glodek, K. McKenney, L.M. Fitzegerald, N. Lee, M.D. Adams, E.K. Hickey, D.E. Berg, J.D. Gocayne, T.R. Utterback, J.D. Peterson, J.M. Kelley, M.D. Cotton, J.M. Weidman, C. Fujii, C. Bowman, L. Watthey, E. Wallin, W.S. Hayes, M. Borodovsky, P.D. Karp, H.O. Smith, C.M. Fraser, and J.C. Venter. 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature*. 388:539–47. doi:10.1038/41483.
- Tsukihara, T., H. Aoyama, E. Yamashita, T. Tomizaki, H. Yamaguchi, K. Shinzawa-Itoh, R. Nakashima, R. Yaono, and S. Yoshikawa. 1996. The whole structure of the 13-subunit oxidized cytochrome *c* oxidase at 2.8 Å. *Science (New York, N.Y.)*. 272:1136–44.
- Varotsis, C., W.H. Woodruff, and G.T. Babcock. 1990. Direct detection of a dioxygen adduct of cytochrome *a*₃ in the mixed valence cytochrome oxidase/dioxygen reaction. *The Journal of biological chemistry*. 265:11131–6.
- Verkhovsky, M.I., I. Belevich, D.A. Bloch, and M. Wikström. 2006. Elementary steps of proton translocation in the catalytic cycle of cytochrome oxidase. *Biochimica et biophysica acta*. 1757:401–7. doi:10.1016/j.bbabbio.2006.05.026.
- Verkhovsky, M.I., J.E. Morgan, A. Puustein, and M. Wikström. 1996. Kinetic trapping of oxygen in cell respiration. *Nature*. 380:268–70. doi:10.1038/380268a0.

- Verkhovsky, M.I., J.E. Morgan, and M. Wikström. 1994. Oxygen binding and activation: early steps in the reaction of oxygen with cytochrome *c* oxidase. *Biochemistry*. 33:3079–86.
- Wagner, A.M., K. Krab, M.J. Wagner, and A.L. Moore. 2008. Regulation of thermogenesis in flowering Araceae: the role of the alternative oxidase. *Biochimica et biophysica acta*. 1777:993–1000. doi:10.1016/j.bbabi.2008.04.001.
- Wikstrom, M.K.F. 1977. Proton pump coupled to cytochrome *c* oxidase in mitochondria. *Nature*. 266:271–273. doi:10.1038/266271a0.
- Wikström, M. 2000. Mechanism of proton translocation by cytochrome *c* oxidase: a new four-stroke histidine cycle. *Biochimica et biophysica acta*. 1458:188–98.
- Wikström, M., and M.I. Verkhovsky. 2002. Proton translocation by cytochrome *c* oxidase in different phases of the catalytic cycle. *Biochimica et biophysica acta*. 1555:128–32.
- Wikström, M., and M.I. Verkhovsky. 2007. Mechanism and energetics of proton translocation by the respiratory heme-copper oxidases. *Biochimica et biophysica acta*. 1767:1200–14. doi:10.1016/j.bbabi.2007.06.008.
- Woodcock, D., D. Crowther, J. Doherty, S. Jefferson, E. DeCruz, M. Noyer-Weidner, S. Smith, M. Michael, and M. Graham. 1989. Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. *Nucleic acids research*. 17:3469–3478. doi:10.1093/nar/17.9.3469.
- Woodruff, W.H., O. Einarsdóttir, R.B. Dyer, K.A. Bagley, G. Palmer, S.J. Atherton, R.A. Goldbeck, T.D. Dawes, and D.S. Kliger. 1991. Nature and functional implications of the cytochrome *a₃* transients after photodissociation of CO-cytochrome oxidase. *Proceedings of the National Academy of Sciences of the United States of America*. 88:2588–92.
- Yoshikawa, S., K. Muramoto, and K. Shinzawa-Itoh. 2011. Proton-Pumping Mechanism of Cytochrome *c* Oxidase.
- Zannoni, D. 1995. Aerobic and anaerobic electron transport chains in anoxygenic phototrophic bacteria. In *Taxonomy and physiology of phototrophic purple bacteria and green sulfur bacteria*. R.E. Blankenship, M.T. Madigan, and C.E. Bauer, editors. Kluwer Academic Publishers, Dordrecht, The Netherlands. 949–971.
- Zufferey, R., O. Preisig, H. Hennecke, and L. Thöny-Meyer. 1996. Assembly and function of the cytochrome *cbb₃* oxidase subunits in *Bradyrhizobium japonicum*. *The Journal of biological chemistry*. 271:9114–9.

Zumft, W.G. 2005. Nitric oxide reductases of prokaryotes with emphasis on the respiratory, heme-copper oxidase type. *Journal of inorganic biochemistry*. 99:194–215. doi:10.1016/j.jinorgbio.2004.09.024.

APPENDIX

A. BACTERIAL GROWTH MEDIA

- **Liquid LB (Luria Bertani) Medium**

Contents	For 1 L
Bacto tryptone	10 g
Yeast extract	5 g
NaCl	10 g

These contents are dissolved in 900 ml dH₂O and pH was adjusted to 7.5 with NaOH. Then the volume is completed to 1 L with dH₂O and sterilized at 121°C for 15-20 minutes.

- **LB plate**

For 1 L liquid LB medium, 15 g agar agar (Merck) was added and sterilized at 121°C for 20 minutes.

- **SOB**

Contents	For 1 L
Bacto tryptone	20 g
Yeast extract	5 g
NaCl	0.584 g
KCl	0.188 g
MgCl ₂ ·6H ₂ O	2.032 g
MgSO ₄ ·7H ₂ O	2.464 g

These contents are dissolved in 1 L dH₂O and sterilized at 121°C for 20 minutes.

- **SOC**

Glucose (20mM of final concentration) was added to the SOB.

- **Liquid MPYE-Medium (Enriched Medium)**

Contents	For 1 L
Bacto peptone	3 g
Yeast extract	3 g
CaCl ₂ (1 mM)	1 ml
MgCl ₂ (1.6 mM)	1 ml

These are dissolved in 800 ml dH₂O and volume completed to 1 L. pH is adjusted with NaOH to 7 and autoclaved at 121°C for 20 minutes.

- **MPYE Plate**

For 1 L liquid MPYE medium, 15 g agar agar (Merck) was added and sterilized at 121°C for 20 minutes.

- **Sistrom's Minimal Medium (Med A)**

Contents	For 1L
NaCl (10%)	5 ml
(NH ₄) ₂ SO ₄ (10%)	5 ml
Sodium (or Potassium) Succinate (10%)	20 ml
L-Aspartic acid (2%, pH: 7)	2 ml
L-Glutamic acid (5%, pH: 7)	2 ml

These contents are complemented up to 1 L and autoclaved at 121°C for 20 minutes. Sterile 10 ml 100X vitamin, 20 ml Potassium phosphate (1M, pH: 6.8), 20 ml Macroelement solution and appropriate antibiotics were added and mixed when the temperature is 50-55°C. Med A plate was prepared by using the same contents however 15 g agar for 1 lt (1.5%) was added before autoclaving.

- **Stock Solution of Med A**

- 1. Microelements Solution**

Contents	For 1L
ZnSO ₄ .7H ₂ O	10.00 g
EDTA	2.50 g
FeSO ₄ .7H ₂ O	5.00 g
H ₃ BO ₃	0.114 g
MnSO ₄ .H ₂ O	1.54 g
CuSO ₄ .5H ₂ O	0.392 g
Co(NO ₃) ₂ .6H ₂ O	0.250 g

These contents were dissolved in 1L dH₂O, autoclaved and stored at +4°C.

- 2. Macro elements Solution (Solution C)**

Contents	For 1L
Nitrilotriacetic acid	10.0 g
MgSO ₄ .7H ₂ O	29.5 g
CaCl ₂ .2H ₂ O	3.3 g
FeSO ₄ .7H ₂ O	0.1 g
Microelements solution	50.0 ml

These contents were dissolved in 800 ml dH₂O. pH was adjusted with KOH to 6.8-7. The volume was completed to 1 L with dH₂O, autoclaved and stored at +4°C.

3. Vitamins (100X)

Contents	For 1L
Nicotinic acid	100.0 mg
Thiamine HCl	25.0 mg
Biotin (40 µg/ml stock)	12.5 ml

These contents were dissolved in 1 L H₂O, sterilized with 0.22 µm filter, and stored at +4°C .

- **Med A Plate**

For 1 L liquid Med A medium, 15 g agar agar (Merck) was added and sterilized at 121°C for 20 minutes.

B. SOLUTIONS AND ANTIBIOTICS

- **Solutions**

- 1. 100mM PMSF**

87 mg Phenylmethanesulfonyl fluoride was dissolved in 5 ml ethanol and isopropanol mixture and stored at +4 °C.

- 2. %20 n-Dodecyl β -D-maltoside (DDM)**

20 g DDM was dissolved in 100 ml ddH₂O and stored at -20°C.

- 3. Acrylamide-Bis Acrylamide Mixture for %11 Laemmli-Type Gel**

30% Acrylamide-Bis Acrylamide(Biorad 161-0159) solution was used.

- 4. 10% SDS**

10 g SDS was dissolved in 100 ml ddH₂O.

- 5. %10 Ammonium persulfate (APS)**

0.1 g Ammonium persulfate (APS) was dissolved in 1ml of ddH₂O, prepared as fresh.

- 6. %5 Milk**

5 g commercial milk protein was dissolved in 100 ml water, prepared as fresh and stored at +4°C during experiment

- 7. Acrylamide-Bis Acrylamide Mixture for %16,5 Schagger -Type Gel**

40 % Acrylamide-Bis Acrylamide (Biorad 161-0148) solution was used.

- 8. 2X Loading Dye for %16,5 Schagger -Type Gel**

6% SDS, 3% β -Mercaptaethanol, 15% Glycerol, 0,025% Bromophenol Blue, 75mM Tris (pH:7)

- 9. 0.25 M Na-Acetate Solution (pH: 5)**

17.01 g Na-Acetate was dissolved in 400 ml ddH₂O. pH was adjusted to 5 with glacial acetic acid. The volume was completed to 500 ml.

10. TMBZ Staining Solution

45 mg TMBZ was dissolved in 30 ml Methanol for 30 mins. In dark, after that 70 ml 0.25 M Na-Acetate solution was added and dissolved for another 30 min.

11. 2 mM Horse Heart Cytochrome c Solution

30 mg horse heart cytochrome c was dissolved in 1 ml Assay Buffer, prepared as fresh.

12. 100mM Sodium Dithiothreitol (DTT)

0.0174 g Sodium Dithiothreitol (DTT) was dissolved in 1 ml 100mM Tris (pH:8), prepared as fresh

13. 20mM Potassium Cyanide (KCN)

0.0130g potassium cyanide was dissolved in 10 ml ddH₂O, prepared as fresh.

14. 20 Mm Myxothiazol

0.97 g Myxothiazol was dissolved in 100 µl DMSO and stored at -20°C.

15. 300 mM TMPD

50 mg TMPD was dissolved in 1 ml Ethanol and stored at -20°C.

- **Antibiotics**

- 1. Ampicillin**

For 10 ml 25 mg/ml stock solution, 0.25 g Ampicillin Sodium Salt (Sigma) was dissolved in 10 ml ddH₂O and sterilized by using 0.22µm filter and stored at -20 °C.

- 2. Tetracycline**

For 40 ml 1.25 mg/ml stock solution, 0.05 g Tetracycline (Sigma) was dissolved in 40 ml 50 % (v/v) ethanol / ddH₂O and sterilized by using 0.22µm filter and stored in dark at -20 °C.

- 3. Kanamycin**

For 10 ml 50 mg/ml stock solution, 0.05 g Kanamycin (Sigma) was dissolved in 10 ml ddH₂O and sterilized by using 0.22µm filter and stored at -20 °C.

- 4. Spectinomycin**

For 5mg/ml stock solution, 0.5 g Spectinomycin (Sigma) was dissolved in 100 ml ddH₂O and sterilized by using 0.22µm filter and stored at -20 °C

C. BUFFERS

- **Transformation Buffer (TB)**

Contents	For 1L
HEPES	0.960 g
CaCl ₂ .H ₂ O	0.880 g
KCl	7.456 g
MnCl ₂ .2H ₂ O	3.520 g

These contents are dissolved in 900 ml dH₂O and pH was adjusted to 6.7 with KOH. Then the volume is completed to 1 L with dH₂O and filter sterilized by using 0.22µm filter and stored at 4°C.

- **TAE Buffer (50X)**

Contents	For 1 L
Trizma Base	242 g
Glacial Acetic Acid	57.1 ml
EDTA (0.5 M pH:8)	100 ml

The volume is completed to 1 L with H₂O, and sterilized by autoclaving.

- **TE**

Contents	For 100 ml
Tris Base (1M, pH: 7.50)	1 ml
EDTA.2H ₂ O (0.5 M, pH: 8)	200 µl

The volume was completed to 100 ml with ddH₂O, and sterilized by autoclaving.

- **MOPS/KCl Buffer (50 mM MOPS/1 mM KCl)**

11. 56 g MOPS and 74 mg KCl was dissolved in 700 ml ddH₂O. pH was adjusted with NaOH to 7.0. The volume was completed to 1L and stored +4 °C.

- **Stacking Gel Buffer for %11 Laemmli-Type Gel**

6.05 g Tris was dissolved in 80 ml ddH₂O. pH was adjusted to 6.8 with HCl. The volume was completed to 100 ml.

- **Separating Gel Buffer for %11 Laemmli-Type Gel**

18.15 g Tris was dissolved in 80 ml ddH₂O. pH was adjusted to 6.8 with HCl. The volume was completed to 100 ml.

- **10X Electrophoresis Buffer for %11 Laemmli-Type Gel**

30 g Tris, 10 g SDS and 144 g Glisin was dissolved in 1L of ddH₂O, used as 1X.

- **10X TBST Buffer for %11 Laemmli-Type Gel**

Content	1L
Tris	30,28 g
NaCl	87,66 g
Triton	5 ml
TWEEN® 20	5 ml

These contents were dissolved in 900 ml ddH₂O. pH was adjusted to 7.5 with HCl. The volume was completed to 1L and used as 1X.

- **5X Transfer Buffer for Western Blotting**

15.14 g Tris and 72,06 g Glisin was dissolved in 1 L ddH₂O. Used as 1X containing 20% methanol.

- **3X Gel Buffer for Western Blotting**

36,33 g Tris and 0,3 g SDS were dissolved in 70 ml ddH₂O. pH was adjusted to 8.5 with HCl. The volume was completed to 100 ml.

- **10X Anode Buffer for %16,5 Schagger -Type Gel**

24,22 g Tris was dissolved in 70 ml ddH₂O. pH was adjusted to 8.5 with HCl. The volume was completed to 100 ml.

- **10X Cathode Buffer for %16,5 Schagger -Type Gel**

12,1 g Tris, 17,92 g Trisine and 0,3 g SDS were dissolved in 100 ml ddH₂O. pH was not adjusted however it should be around 8.25.

- **Enzyme Activity Assay Buffer**

0.605 g Tris and 3.7 g KCl were dissolved in 400 ml ddH₂O. pH was adjusted to 7.0 with HCl. The volume was completed to 500 ml and sterilized with 0.22µm filter and stored at +4°C

- **HEPES Buffer**

11.18 g KCl, 9.7 g KSCN and 120 g HEPES were dissolved in 800 ml ddH₂O. pH was adjusted to 7.3 with KOH. The volume was completed to 1L ml and sterilized with 0.22µm filter and stored at +4 °C.

D. CHEMICALS

30% Acrylamide-Bis Acrylamide (Biorad, 161-0159)

40 % Acrylamide-Bis Acrylamide (Biorad, 161-0148)

Agar (Merck, 101614)

Agarose (Sigma, 9012-36-6)

Ammonium sulfate[(NH₄)₂SO₄] (Sigma, 7783-20-2)

Ammonium persulfate [APS] (Sigma, A-9164)

Ampicillin (Sigma, A-0166)

L-Aspartic acid (Sigma, A,-9256)

Bacto peptone (Merck, 884113)

Bacto tryptone (Merck, 667114)

Boric acid [H₃BO₃] (Sigma,10043-35-3)

Biotin (Sigma,58-85-5)

Bromophenol Blue (Sigma, 115-39-9)

Calcium Chloride [CaCl₂] (Merck, 208290)

Calcium chloride dihydrate [CaCl₂.2H₂O] (Sigma,10035-04-8)

Cobalt(II) nitrate hexahydrate [Co(NO₃)₂.6H₂O](Sigma,10026-22-9)

Copper(II) sulfate pentahydrate [CuSO₄.5H₂O] (Sigma,7758-99-8).

EDTA [Ethylenediaminetetraacetic acid] (Sigma,60-00-4)

EtBr [Ethidium Bromide] (Sigma, E-8751)

Ferrous Sulfate heptahydrate [FeSO₄.7H₂O] (Sigma,7782-63-0)

Glucose (Merck, 346351)

Glycerol, cell culture tested (Sigma, G-2025)

Glycerol, Electrophoresis grade (Sigma G-8773)

L-Glutamic acid (Sigma, G-1251)

n-Dodecyl β-D-maltoside [DDM] (Sigma,69227-93-6)

HEPES (Sigma,7365-45-9)

Horse Heart Cytochrome c (Sigma, 9007-43-6)

Hydrogen peroxide [(H₂O₂), 30%] (Aldrich, Chemical Co., 21, 676.3)

Kanamycin (Sigma, K-4378)

2-Mercaptoetanol (Electrophoresis Regent, Sigma, M-7154)

Methanol (Carlo Erba reagent, 309203)

Magnesium chloride[MgCl₂] (Merck, 442615)

Magnesium chloride hexahydrate[MgCl₂.6H₂O] (Sigma, 7791-18-6)

Magnesium sulfate heptahydrate[MgSO₄.7H₂O](Sigma, 10034-99-8)

Manganese(II) sulfate monohydrate [MnSO₄.H₂O] (Sigma,10034-96-5)

MOPS (Sigma,1132-61-2)

Myxothiazol (Sigma, 76706-55-3)

α-Naphthol (Sigma, N-1000)

Nicotinic acid (Sigma,59-67-6)

Nitrilotriacetic acid (Sigma,139-13-9)

Phenylmethanesulfonyl fluoride [PMSF] (Sigma,329-98-6)

Potassium chloride [KCl] (Sigma, 7447-40-7)

Potassium cyanide [KCN] (Sigma,151-50-8)

Potassium thiocyanate [KSCN] (Sigma,333-20-0)

Sodium acetate (Merck, 6268)

Sodium chloride [NaCl] (Merck, 106404)

Sodium Dithiothreitol [DTT] (Sigma,3483-12-3)

Sodium dodecyl sulfate [SDS] (Sigma,151-21-3)

Sodium hydroxide [NaOH] (Merck, 106462)

Spectinomycin (Sigma,S0692)

Succinic acid [Disodium Salt Hexahydrate] (Sigma, S-9637)

Tetracycline Hydrochloride, Crystalline (Sigma, T-33839 105)

N,N,N',N'-Tetramethyl-p-phenylenediamine [TMPD] (Sigma,100-22-1)

N-(3-Sulfopropyl)-3,3',5,5'-tetramethylbenzidine sodium salt [TMBZ] (Sigma, 102062-46-4)

Thiamine hydrochloride (Sigma,67-03-8)

Trizma® base(Sigma,77-86-1)

Tricine (Electrophoresis Grade) Sigma T-7911

Triton (Sigma, N150)

TWEEN® 20 (Sigma,9005-64-5)

Yeast Extract (Merck, 103750)

Zinc sulfate heptahydrate[ZnSO₄.7H₂O] (Sigma, 7446-20-0)

E. ENZYMES

1. Restriction Endonucleases

<i>Alw441</i> (MBI)	: cat. # ER0041
<i>DpnI</i> (MBI)	: cat.# ER1705
<i>EcoRI</i> (MBI)	: cat. # ER0275
<i>HindIII</i> (MBI)	: cat. # ER0505
<i>KpnI</i> (New England Biolabs)	: cat. # R0142S
<i>SacI</i> (MBI)	: cat. # ER1135
<i>SphI</i> (<i>PaeI</i>) (MBI)	: cat. # ER0601
<i>SpeI</i> (New England Biolabs)	: cat. # R0133S
<i>XhoI</i> (MBI)	: cat # ER0695
Fast Digest <i>XhoI</i> (MBI)	: cat. # FD0694

2. Polymerases

<i>Pfu</i> DNA Polymerase (MBI)	: cat. # EP0501
<i>PfuUltra</i> High-Fidelity DNA Polymerase (Stratagene)	: cat.#600380

3. Ligases

T4 DNA Ligase (MBI)	: cat. # EL0335
T ₄ DNA Ligase (Stratagene)	: cat. #: 600011

4. Phosphatases

Shrimp Alkaline Phosphatase (SAP) (MBI)	: cat. # ER EF0341
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5. dNTP Set

dNTP (MBI)	: cat. # R0181
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F. EQUIPMENTS USED IN THIS STUDY

PCR(Technne, TC 3000)

34°C and 37°C Incubators (Nuve EN 500, Nuve FN 500)

34°C and 37°C shaker-incubator (Gerhardt)

Shaker- Heidolph Unimax 2010

Electrophoresis Systems (Thermo Scientific)

DNA Electrophoresis System

Power supply (Thermo EC250-90)

Dry block (VWR Digital Dry-Block)

Shaker-heater (IKA RCT basic)

Autoclave (Hirayana)

pH meter (HANNA HI 221)

Micropipettes (Finnpipette)

0,5-10, 1-10, 10-100, 100-1000 µl and 1- 10 ml pipettes

Desktop centrifuges (Hettich Micro 120)

Centrifuge (Hettich Rotina 38R)

+4°C refrigerators (Arçelik)

-20°C deepfreeze (Arçelik)

-80°C deepfreeze (Thermo Scientific)

-80°C deepfreeze (Biolabs)

Spectrophotometer (HITACHI U-1900)

UV Transilluminator (UVP)

Imaging system (UVP Photo Doc-It™)

Vortex (Yellowline TTS2)

Water Purification System (Human Corporation)

CURRICULUM VITAE

GÜLGEZ GÖKÇE YILDIZ

Date of Birth, Place: 10.07.1980, Ankara-TURKEY

Contact Address: Abant İzzet Baysal University, Faculty of Arts and Sciences, Department of Biology, 14280, Bolu TURKEY.

Telephone: +90-374-2541000-2714/2742

Fax: +90-374-2534642

e-mail: gulgez_g@ibu.edu.tr, gulgezg@gmail.com



Educational Status:

Doctor of Philosophy: Abant İzzet Baysal University (AIBU), Dept. of Biology, 2006-

Master of Science: Abant İzzet Baysal University (AIBU), Dept. of Biology, 2003-2006

Bachelor of Science: Abant İzzet Baysal University (AIBU), Dept. of Biology, 1999-2003

High School and Secondary School: Ankara Atatürk High School 1992-1998

International Experience:

2005: St. Cyril and Methodius University, Institute of Biology, Skopje, Republic of Macedonia; research on project-10 days.

2010-2011: University of Pennsylvania, Department of Biology, PA, USA; researcher-4 months. University of Illinois at Urbana-Champaign, Department of Biochemistry, IL, USA; researcher-8 months.

Academic Qualifications:

2004- : Abant İzzet Baysal University, Dept. of Biology, Research Assistant.

Publications:

Thesis:

M.S.

Studies on Shelf-Life Extension of Bread By a Cellulase Bearing Enzyme Complex from Anaerobic Fungi, AIBU, Department of Biology, 2006

Papers (SCI-Expanded Cited and the other) :

Regular Papers

1. Mozafari, M.R., Khosravi-Darani K., **Borazan, G.G.**, Cui, J., Pardakthy, A., Seyhun Yurdugül. Encapsulation of Food Ingredients Using Nanoliposome Technology, International Journal of Food Properties, 11, 833-844, 2008 (Review).
2. **Gülgez Gökçe Yıldız**, Mehmet Öztürk and Belma Aslım, Identification of Lactobacillus strains from breast-fed infant and investigation of their cholesterol-reducing effects, World Journal of Microbiology and Biotechnology, Volume 27, Number 10, Pages 2397-2406, 2011.
3. Mehmet Öztürk, Şükriye Er, **Gülgez Gökçe YILDIZ**, Replacement of the Glu380 with Gln380 in subunit I of cytochrome *cbb₃* oxidase from *Rhodobacter capsulatus* results in inactive enzyme, Turkish Journal of Biochemistry, 37 (1); 48–53, 2012.
4. Seyhun Yurdugul, Natalija-Atanasova Pancevska, **Gülgez Gökçe Yildiz**, Faruk Bozoglu, The Influence Of A Cellulase Bearing Enzyme Complex From Anaerobic Fungi On Bread Staling, Romanian Agricultural Research, No 29, Pages 271-279, 2012.

Conference Papers

1. **Gülgez Gökçe YILDIZ**, Mehmet ÖZTÜRK, Belma ASLIM, Identification Of Human Originated *Lactobacillus* Strains And Investigation of Their Cholesterol Reducing Effects, Turkish Journal of Biochemistry, 34, Special Issue 1, 86-87, 2009.
2. Mehmet ÖZTÜRK, Davut ERDOĞAN, **Gülgez Gökçe YILDIZ**, Şükrüye ER, Characterization of Three Conserved Tyrosine Mutants of Cytochrome *cbb₃* Type Oxidase in *Rhodobacter capsulatus*, Turkish Journal of Biochemistry, 34, Special Issue 1, 90, 2009.
3. Mehmet ÖZTÜRK, **Gülgez Gökçe YILDIZ**, Davut ERDOĞAN, Characterization of Conserved Amino acid Mutantions in *cbb₃* Type Oxidase Enzyme of *Rhodobacter capsulatus*, Cell Membranes and Free Radical Research, Volume 2, Number 1, 19-20, 2010.

4. **Gülgez Gökçe Yildiz**, Mehmet Öztürk, Fevzi Daldal, Proton input channel of cytochrome *cbb₃* type oxidase in *Rhodobacter capsulatus* , Current Opinion in Biotechnology, Volume 22, Supplement 1, Page S80, 2011

Letters

1. Ranjani Murali, **Gökçe G. Yildiz**, Fevzi Daldal, Robert B. Gennis, Stoichiometry of proton pumping by the *cbb₃*-type oxygen reductase in whole cells of *Rhodobacter capsulatus* at pH 7 is about 0.5 H⁺ per electron, Proceedings of the National Academy of Sciences, Volume 109, No 32, Page E2144, 2012.

Oral Presentations:

Seyhun YURDUGÜL, Natalija ATANASOVA-PANCEVSKA , Gülgez Gökçe YILDIZ, Dzoko KUNGULOVSKEI , and Faruk BOZOGLU , Retardation Of Staling In Bread By A Cellulase Containing Enzyme Complex, Partially Isolated From Cellulose Degrading Anaerobic Fungi, 5th International Symposium of Anaerobic Microbiology, June 21-24, 2007, Domzale, SLOVENIA.

Gülgez Gökçe YILDIZ, Mehmet ÖZTÜRK, Belma ASLIM, Identification Of Human Originated *Lactobacillus* Strains And Investigation of Their Cholesterol Reducing Effects, 21th National Biochemistry Congress, October 29-31, 2009, İstanbul, TURKEY.

Mehmet ÖZTÜRK, Davut ERDOĞAN, Gülgez Gökçe YILDIZ, Şükrüye ER, Characterization of Three Conserved Tyrosine Mutants of Cytochrome *cbb₃* Type Oxidase in *Rhodobacter capsulatus*, 21th National Biochemistry Congress, October 29-31, 2009, İstanbul, TURKEY.

Mehmet ÖZTÜRK, **Gülgez Gökçe YILDIZ**, Davut ERDOĞAN, Characterization of Conserved Amino acid Mutations in *cbb₃* Type Oxidase Enzyme of *Rhodobacter capsulatus*, 3rd International Congress on Cell Membranes and Oxidative Stress: Focus on Calcium Signaling and TRP Channels, June 22-27, 2010, Isparta, TURKEY.

Gölgez Gökçe Yıldız, Mehmet Öztürk, Fevzi Daldal, Proton input channel of cytochrome *cbb₃* type oxidase in *Rhodobacter capsulatus*, European Biotechnology Congress, September 28- October 1, 2011, İstanbul, TURKEY.

Gölgez Gökçe Yıldız, Mehmet Öztürk, Robert B. Gennis, Fevzi Daldal, *Rhodobacter capsulatus*'un *cbb₃*-Tipi Oksidaz Enzimindeki Kısmi Olarak Korunmuş Aminoasit Mutasyonlarının Karakterizasyonu, 2. Ulusal Moleküler Biyoloji ve Biyoteknoloji Kongresi, 15-18 Kasım 2012, Antalya, TÜRKİYE.

Poster Presentations:

Gölgez Gökçe YILDIZ, Mehmet ÖZTÜRK, Characterization of three conserved amino acid mutations in *cbb₃*-type oxidase enzyme of *Rhodobacter capsulatus*. ESF Functional Genomics Workshop: PROTIST '10 - Novel Approaches in Protein Engineering, April 23-25, 2010, İstanbul, TURKEY.

Completed / Ongoing Projects:

International

Yurdugöl, S., Bozoğlu, F., **Yıldız, G.G.**, Çelekli, A., Kungulovski, D., Isolation and Characterization of Cellulolytic Enzymatic Complex of Anaerobic Fungi for Extending the Shelf Life of Bread, Turkish Scientific and Technical Research Council(TUBITAK)-TBAG-U-73 (103 T 084)-Republic of. Macedonia Ministry of Science and Education Joint Project 2003-2007 (Completed)

National

Öztürk, M., **Yıldız, G. G.**, Identification Of Human Originated *Lactobacillus* Strains And Investigation of Their Bile Salt Hydrolase (BSH) enzyme activities and Cholesterol Reducing Effects. AIBU-Scientific Research Project, Code #: 2008.03.01.284 2008-2010 (Completed)

Öztürk, M., Er, Ş., **Yıldız, G. G.** Investigation of the K-and D-Proton Pumping Channels of *cbb₃*-type oxidase in *Rhodobacter capsulatus* Turkish Scientific and Technical Research Council(TUBITAK)- TBAG (107T519) 2007-2011 (Completed)

Courses Taken:

Modern Techniques in Biochemistry and Molecular Biology (Summer School for Graduated Students), Ege University, Dept of Biochemistry (Turkey), Gülhane Military Medical Acedemy (Turkey), Atatürk Universty (Turkey), TU, Berlin, Dept. Biochemistry (Germany), 17-27 June 2007, Muğla, TURKEY

Protein Analytics (Summer School for Graduated Students), Ege University, Dept of Biochemistry (Turkey), Ankara University (Turkey), Erzincan Univesity (Turkey), Regensburg University (Germany), 20-26 September 2009, Muğla, TURKEY.

Scholarship/Fellowship:

TÜBİTAK-BİDEB 2214-International Research Fellowship Programme for PhD students, full term fellowship, 12 months (June 2010- July 2011).

Courses taught at AIBU:

Biochemistry Laboratory I-II, Chromosome Techniques Laboratory, Food Microbiology Laboratory, General Biology Laboratory I-II, Industrial Microbiology Laboratory, Medical Microbiology Laboratory, Microbiology Laboratory, Molecular Biology Laboratory.

Language Skills:

Turkish (native), English (good)