

**SITE- DIRECTED MUTAGENESIS OF HIGHLY CONSERVED
RESIDUES IN HELIX IX OF SUBUNIT I OF CYTOCHROME *cbb*₃
OXIDASE FROM *RHODOBACTER CAPSULATUS***

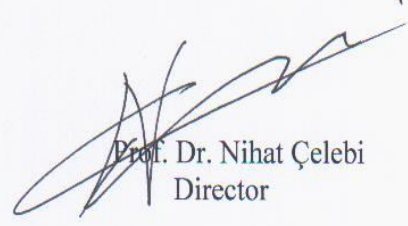
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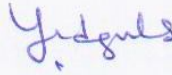
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ABSTRACT

SITE- DIRECTED MUTAGENESIS OF HIGHLY CONSERVED RESIDUES IN HELIX IX OF SUBUNIT I OF CYTOCHROME *cbb*₃ OXIDASE FROM *RHODOBACTER CAPSULATUS*

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Oxygen reductases are terminal respiratory oxidases in mitochondria and many aerobic bacteria and archaea. Site-directed mutagenesis experiments and the crystal structures of the *aa*₃-type oxygen reductases revealed two proton transfer pathways, named the D- and the K-pathways. Aspartic acid 361 (D361), glutamate 380 (E380) and lysine 387 (K387) are highly conserved amino acid residues (*R. capsulatus* numbering) in the subunit I of the *aa*₃ and *ba*₃-type oxygen reductases. Mutations at glutamate 380 (E380) locus have been shown to block the oxygen reduction and the uptake of protons, suggesting that this residue plays an important role in the catalytic mechanism of *aa*₃ type oxidases. D361

and K387 are also thought that highly conserved amino acids especially in *aa*₃ type oxidase and play important roles on enzyme activity.

Moreover, thermodynamic and spectroscopic properties of the redox centers in oxygen reductases indicated that these three residues were important for histidine-glutamate/aspartate structural motifs found in a variety of enzymes. A sequence comparison of the catalytic subunits shows that these residues are not conserved within VIII. helix of *cbb*₃-type oxygen reductase. However, homology modeling has suggested that D361, E380 and K387 are located within helix IX of subunit I and fully conserved among the *cbb*₃-type oxygen reductases, might fulfill this function.

In the present study, in order to understand molecular mechanism of proton pumping channel, conserved aspartic acid, glutamic acid and lysine residues within helix IX of subunit I of the *cbb*₃-type oxygen reductases from *R. capsulatus* were substituted for asparagine 361 (N361), glutamine 380 (Q380) and methionine 387 (K387) respectively by site directed mutagenesis. The effects of these mutations on enzyme activity were evaluated by NADI staining and it was found that while the E380Q mutant was completely inactive, other D361N and K387M mutant had wild type activity. The result supports the proposal that the *cbb*₃-type oxygen reductases have a proton-conducting channel that is analogous to the D-channel in the *aa*₃-type oxygen reductases.

Keywords: Heme-copper oxidase, cytochrome *cbb*₃-type oxidase reductases, proton pumping channels, site-directed mutagenesis, *Rhodobacter capsulatus*.

ÖZET

RHODOBACTER CAPSULATUS' TA BULUNAN *cbb*₃ OKSİDAZIN I. ALT ÜNİTESİNİN IX. HELİKSİ ÜZERİNDEKİ OLDUKÇA KORUNMUŞ REZİDÜLERİNİN YÖNLENDİRİLMİŞ MUTAGENEZİ

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Oksijen redüktazlar; mitokondri, birçok aerobik bakteri ve arke'lerin solunum zincirinin son halkasını oluşturmaktadır. Yönlendirilmiş mutageniz deneyleri ve *aa*₃-tipi oksijen redüktazların kristal yapıları oksijen redüktazların D- ve K- kanalı olarak adlandırılan iki proton transfer kanalına sahip olduğunu göstermektedir. Aspartik asit 361 (D361), glutamat 380 (E380) ve lizin 387 (K387) *aa*₃ ve *ba*₃-tipi oksijen redüktazların I. alt ünitelerinde (numaralandırma *R. capsulatus*' a göre) oldukça korunmuş olarak bulunan amino asitlerdir. 380 glutamat bölgesindeki mutasyonlarının oksijenin indirgenmesi reaksiyonu ve proton alınımını durdurması, bu rezidünün *aa*₃ tip oksidazların katalitik mekanizmasında önemli rol oynadığını göstermektedir. D361 and K387 amino asitlerinin de özellikle *aa*₃ tip oksidazlarda oldukça korunmuş olduğu ve enzim aktivitesi üzerinde önemli roller oynadığı düşünülmektedir.

Bunun dışında oksijen redüktaz'ların redoks merkezinin spektroskopik ve termodinamik özellikleri, bu üç rezidünün birçok enzimlerde de görülen histidine/glutamat/aspartat yapısal motifi için önemli olduğunu ortaya koymaktadır. Ancak katalitik alt ünitelerin amino asit dizi analizleri bu rezidülerin *cbb*₃-tipi oksijen redüktazların VIII. heliks'inde korunmadığını göstermektedir. Bununla beraber, homoloji modellemeleri *cbb*₃-tipi oksijen redüktazların I. alt ünitesinin IX. heliks'inde tamamen korunmuş olan D361, E380 ve K387' nin bu fonksiyonu yerine getirdiğini düşündürmektedir.

Bu çalışmada ayrıca proton pompalama kanalının moleküler mekanizmasını anlamak için, *R.capsulatus*'tan elde edilen *cbb*₃-tipi oksijen redüktaz'ın I. alt ünitesindeki IX. heliks'te yer alan korunmuş olan aspartik asit, glutamat ve lizin amino asitleri yönlendirilmiş mutagenез tekniği kullanılarak sırasıyla asparjin (N), glutamin (Q) ve metiyonin (M)' e dönüştürülmüştür. Mutasyonun enzim aktivitesi üzerindeki etkisi NADİ boyama ile tayin edilmiş ve E380Q mutantının tamamıyla inaktifken, D361N ve K387M mutantlarının yaban tip enzim aktiviteye sahip oldukları bulunmuştur. Sonuçlar *cbb*₃-tipi oksijen redüktazların *aa*₃-tipi oksijen redüktazlardaki D-kanalına benzer bir proton iletim kanalına sahip olduğu önerisini desteklemektedir.

Anahtar Kelimeler: Demir-bakır oksidazlar, *cbb*₃-tipi oksijen redüktazlar, proton pompalama kanalları, yönlendirilmiş mutagenез, *Rhodobacter capsulatus*.

to my family

.

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ABBREVIATIONS

Chemical compounds:

ADP: Adenosine diphosphate

ATP: Adenosine triphosphate

Fe/S: Iron-sulfur cluster

FAD: Flavin adenine dinucleotide

FMN: Flavin mononucleotide

COOH: Carboxyl group

SAP: Shrimp alkaline phosphatase

Amp^R: Ampicilline resistance

IPTG: Isopropyl – β -D-thiogalactopyranoside

Kan^R: Kanamycin resistance

NH₂: Amino

NAD: Nicotinamide adenine dinucleotide

NADH: Nicotinamide adenine dinucleotide, reduced form

NADP: Nicotinamide adenine dinucleotide phosphate

NADPH: Nicotinamide adenine dinucleotide phosphate, reduced form

NAD⁺/NADH: Nicotinamide adenine dinucleotide [oxidized/reduced]

UQ/UQH₂: Ubiquinone/ubiquinol

LB: Luria Bertani Media

PEG: Polyethylene glycol

SDS: Sodium dodecyl sulfate

Tet^R: Tetracycline resistance

Tris: Tris (hydroxymethyl) aminomethane

X-gal: 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

D: Aspartic acid (Asp)

E: Glutamic acid (Glu)

K: Lysine (Lys)

Redox centers of cytochrome *c* oxidase:

Cu_A: Dinuclear copper center in subunit II

Cu_B: Mononuclear copper center in subunit I

Heme *a*: Low-spin heme in subunit I

Heme *a*₃: High-spin heme in subunit I, oxygen binding site

Fe_a: The iron ion of heme *a*

Fe_a₃: The iron ion of heme *a*₃

States of cytochrome *c* oxidase:

A ferrous-oxy: Intermediate

F ‘ferryl’: Intermediate

P ‘peroxy’: Intermediate

Others:

kbp: Kilo base pair

Cyt: Cytochrome

ETC: Electron transfer chain

ICM: Intracytoplasmic membranes

bp: Base pair

kDa: Kilo Dalton

MCS: Multiple cloning sites

ORF: Open reading frame

PAGE: Polyacrylamide–gel electrophoresis

PC: Plastocyanin

PCR: Polymerase chain reaction

PQ: Plastoquinone

Q: Quinone pool

QA and QB: Primary and secondary quinone acceptors of the RC, respectively.

Qo: Ubihydroquinone oxidizing site of the cytochrome *bc*₁ complex

Qox: Quinol oxidase

RC: Photochemical reaction center

Rc and Rs: *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*, respectively.

Res: Respiratory

RT: Room temperature

TM: Transmembrane helix

UV: Ultraviolet

v/v: volume/volume

w/v: weigh/volume

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CHAPTER 1

INTRODUCTION

1.1. The Bioenergetics

The bioenergetics is a field study of the flow and transformation of energy in and between living organisms and their environment. Life is comprised of a complex set of inter- and intra- cellular physical interactions and chemical reactions.

These life processes produce order, generate and maintain complex structures, all of which require energy. This drives the self-organization of the open systems and prevents it from reaching thermodynamic equilibrium [1]. The first signs of life can be traced back to 3.5 billion years ago. Energy transduction of these organisms was probably based on the oxidation of simple inorganic compounds, such as NO, H₂, H₂S, NH₃, and CH₄, which were present in the Earth's early atmosphere [2].

Photosynthesis and respiration have formed ever since the main energy transduction systems on Earth. ATP is at the universal "currency" of chemical energy; it is found in all type organisms and must have occurred in the earliest life-forms. Living things can use ATP like a battery. ATP is synthesized by three metabolic pathways; photosynthesis, respiration and fermentation. At a first glance, photosynthesis and aerobic oxidation appear to have little common.

However, a revolutionary discovery in cell biology is that bacteria, mitochondria and chloroplast all use the same (or very nearly same) process, called **chemiosmosis** (or chemiosmotic coupling), to generate ATP from ADP and Pi. The membrane plays a crucial role in this mechanism by forming a closed system across which the proton concentration gradient can be maintained. The electrochemical proton gradient is formed by two components; a difference in proton concentration across the membrane, plus a difference in electrical potential. The controlled flow of protons down the gradient can do work, such as to drive ATP synthesis. This is called the **chemiosmotic theory**, a mechanism for the coupling of electron transfer through a series of carriers to ATP synthesis, first proposed by Peter Mitchell [3]. The immediate energy sources for constructive cellular processes that power ATP synthesis are the membrane proton concentration gradient and electric potential (voltage gradient), collectively termed the **proton-motive force**. The proton-motive force is generated by the stepwise movement of electrons from higher to lower energy states via membrane-bound **electron-carriers**. The membrane-bound nature of the electron-transfer processes is critical, as the free energy made available in exergonic redox chemistry is used to drive ATP formation. The mechanism of these intricately coupled reactions and their coupling to the proton-pumping machinery of the enzyme is one of the key problems in bioenergetics.

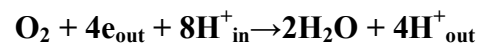
Aerobic respiration plays a fundamental role in Earth's biogeochemical oxygen cycle. It has been estimated that ~75% of the O₂ produced by oxygenic

photosynthesis is reduced to water via this enzymatically catalyzed process, tightly coupling two of the most widespread metabolisms on earth. Aerobic respiration is also the most exergonic metabolism known and appears to be a requirement for multicellular life. Respiration is performed by a series of integral membrane protein complexes that form electron transfer chains, found within the inner mitochondrial membrane of aerobic eukaryotes and the cytoplasmic membrane of many prokaryotic organism [4,5]. Mitochondria have a linear electron transfer chain terminating with cytochrome *c* oxidase, a proton-pumping oxygen reductase which reduces O₂ to water. Prokaryotes have more complicated electron transfer chains with branches leading to different terminal electron acceptors (e.g., fumarate, nitrate, Fe₃⁺, O₂), allowing for metabolic flexibility when different environments are encountered [6].

1.1.1. The Respiratory Chain

The respiratory chain (also known as the electron transfer chain=ETC) catalyzes the final steps of cellular respiration [7] and couples a chemical reaction between an electron donor (such as NADH) and an electron acceptor (such as O₂) to the transfer of H⁺ ions across a membrane, through a set of mediating biochemical reactions. In eukaryotes, the electron transport chain can be found in the inner mitochondrial membrane (Fig. 1.1) where it serves as the site of oxidative phosphorylation. It is also found in the thylakoid membrane of the chloroplast in photosynthetic organisms. In bacteria and archaea, the electron transport chain is located in their cell membrane. The mammalian enzyme

consists of at least 42 different subunits [3]. ETC possesses at least 18 discrete electron carriers, mainly organized into transmembrane enzyme complexes [known as Complexes I, III, and IV] and two mobile carriers circulating between and connecting these three complexes [ubiquinone and cytochrome *c*] [8]. Compared to mitochondrial respiratory chain, bacterial respiratory chain is more complicated and has branch sites [8].



Complex IV (cytochrome *c* oxidase), is the last enzyme of the mitochondrial respiratory chain, is the terminal complex in the respiratory chain that transfers electrons to the ultimate acceptor – O_2 – reducing it to H_2O [8]. It removes four electrons from four molecules of cytochrome *c* and transfers them to molecular oxygen (O_2), producing two molecules of water (H_2O). Cytochrome *c* oxidase also moves two electrons across the inner mitochondrial membrane, adding to the proton gradient.

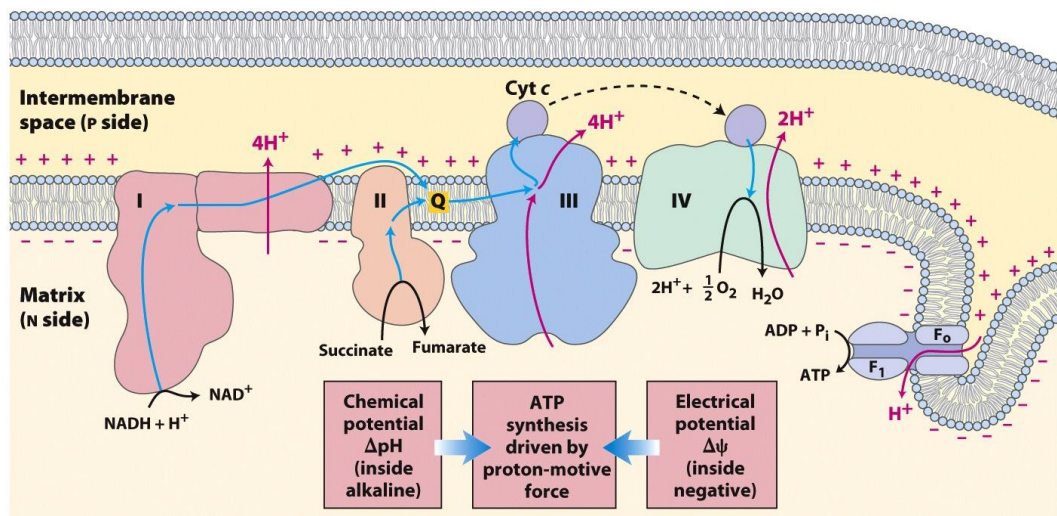
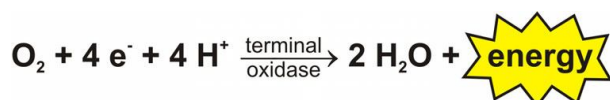


Figure 1. 1 The respiratory chain in the mitochondria.

1.2. The Terminal Oxidases

1.2.1. Overall Function

The terminal oxidases of membrane-bound electron transfer chains from aerobic organisms catalyse the reduction of dioxygen to water, illustrate a number of the thermodynamic and structural principles that have driven the development of respiration [4, 9]. For the O₂ reduction four electrons and four protons are required. Electrons are supplied one by one by cytochrome *c* from outer side of the membrane, is called the P-side (the positively charged side of the mitochondrial inter-membrane space and bacterial periplasmic side). At the same time, four protons are taken up from inner-side of the membrane, is called the N-side (negatively charged side of the mitochondrial matrix and bacterial cytoplasm) [8,10]. The overall reaction of the O₂ reduction by terminal oxidase is:



The function of the terminal oxidase is to bind, activate O₂, reduce it to H₂O, and to conserve the energy released upon its reduction. In order to activate O₂, the minimal requirement for the enzyme is that it should contain a metallic O₂⁻ binding center and the metal should have at least two redox states in order to donate electrons to O₂ and after that get reduced. The energy of the O₂ reduction is transformed in the form of the electrochemical transmembrane potential. The membrane potential generated by the terminal oxidase is created by two different mechanisms: [i] vectorial transfer of electrons from the P-side and protons from the N-side to the O₂ reduction center, resulting in translocation of one net charge

across the membrane for each e^- ; and [ii] most terminal oxidases pump H^+ [translocate H^+ against the proton concentration gradient from the N- to the P-side] for each e^- . In the last case, altogether, two net charges are translocated across the membrane for each e^- . In total, four protons are pumped by most of the terminal oxidases for each O_2 molecule reduced [8].

1.2.2. The Family of Heme-Copper Oxidases

The origin and evolution of respiratory O_2 reductases necessarily is linked to the history of O_2 in the biosphere. Oxygen reductases were identified by [11], and since then much attention has been dedicated to their study with remarkable landmarks [12]. Attempts to use enzyme phylogenies to gather information on this link date back to the mid-1990s when the group of Matti Saraste started to analyse the phylogeny of the major class of O_2 reductases, the so-called heme-copper oxidases (HCO) [2]. These early studies identified three distinct subfamilies of HCOs and the obtained phylogenetic trees suggested the presence of HCOs in the common ancestor of Bacteria and Archaea, *i.e.* prior to photosynthetic oxygen release. A more recent phylogenetic study [4] of HCOs reported evidence for extensive horizontal gene transfer (HGT) and concluded on a late origin of HCOs followed by horizontal distribution over the two prokaryotic domains [13]. HCOs is the largest family members of terminal oxidases according to structure and ability to pump protons which translocate H^+ , additionally to vectorial transfer of protons for the O_2 reduction [13] and they are

the terminal enzyme in the respiratory chains from bacteria, archaea and eukarya [14].

The superfamily of HCOs is defined by the primary sequence of their catalytic subunit, which is composed of twelve transmembrane helices with six highly conserved histidines ligating three cofactors; a high-spin heme and a copper ion, Cu_B in the unique catalytic site (also known bimetallic centre and gives this family its name) and an additional low-spin heme. A redox active tyrosine residue covalently bound to one of the copper ligands [3, 4, 15, 16, 17, 18]. The tyrosine is a key residue for the catalysis, donating an electron and a proton for the first steps of oxygen reduction chemistry [17].

Oxidase members of the superfamily vary in the type of electron donor (cytochrome *c*, quinol, or high-potential iron-sulfur protein), the types of hemes (A, B, or O), and the number of subunits present in the enzymatic complex (ranging from 2, in some bacteria, to as many as 13 in mammalian mitochondria) [10]. Therefore, the presence of these six histidines is diagnostic for members of the superfamily.

The oxygen reductases are multisubunit integral membrane complexes and have been previously classified into three main families based on structural analyses: (i) type A (mitochondrial-like oxidases), (ii) type B (*ba*₃-like oxidases) and (iii) type C (*cbb*₃-type oxidases) families of oxygen reductases [4]. More recent work has identified five additional oxygen reductase families: the D-, E-, F-, G-, and H-families [19]. The first group can be further divided into two subfamilies according to the presence (type A1) or absence (type A2) of a conserved glutamate in transmembrane helix VI at the hydrophobic end of one of the proton pathways (the so-called D-channel): (i) type A1 (E containing

oxidases), comprising the enzymes with a glutamate residue in the motif – XGHPEV-, and (ii) type A2 (YS containing oxidases), enzymes having instead a tyrosine and a serine in the alternative motif -YSHPXV-[4, 7]. It appears that enzymes from each of the eight oxygen reductase families couple the reduction of oxygen to proton pumping and the generation of a transmembrane voltage [19].

The A family of oxygen reductases is the most studied and largest group of the enzymes in the heme-copper superfamily. Examples of heme-copper oxidases that belong to subgroup A1 are the mitochondrial cytochrome *c* oxidase and the closely related enzymes from bacteria such as *Paracoccus denitrificans* and *Rhodobacter sphaeroides*, as well as the ubiquinol oxidase from *Escherichia coli*. Type B oxidases are a heterogeneous group, which have low sequence homology with the mitochondrial cytochrome *c* oxidase. The characteristic examples are *T. thermophilus* [12,20], *aa₃* from *Acidianus ambivalens* [12]. The third group, type C, differs substantially from both other types of heme-copper oxidases with respect to the amino acid composition. The group consists only of *cbb₃* oxidases [7]. The *c* type cytochromes are probably among the most popular proteins for biochemical studies and have been the subject of several review books [21]. The C-family (*cbb₃* oxidases) is the second most abundant oxygen reductase family after the A-family, making up more than 20% of the sequences of the heme-copper superfamily [19]. Cytochrome *c* oxidase from *R. sphaeroides* is one of the best characterized terminal oxidases in respect to function [22]. The combination of site-directed mutagenesis and time-resolved spectroscopic techniques has unveiled important functional properties of this enzyme, such as the involvement of specific residues in the catalytic reaction, pathways through the enzyme used for the transfer of substrate or pumped protons, and the coupling

mechanisms of electron and proton transfer in specific reactions steps [23]. However, the proton-pumping mechanism has yet to be revealed [24]. Amino acid sequence comparisons and structural alignments clearly show that specific amino acids are not universally present for all heme-copper oxidases except, a tyrosine, covalently bound to histidine despite it is being in a different position [12].

Oxygen reductase (respiratory oxidase) and nitric oxide reductase show links between the nitrogenic and the oxygenic respiratory pathways. Subunit I of the heme-copper terminal oxidase superfamily and the largest subunit, NorB, of this nitric oxide reductase complex are homologous [25]. On the other hand, there is suggestion the aerobic respiratory chain evolved from an ancestral terminal respiratory enzyme as NO reductase in the anaerobic respiratory chain, accompanying incorporation of copper [26].

1.2.3. Cytochrome *c* Oxidase in *Rhodobacter capsulatus*

C-type cytochromes, one or more heme group (protoheme IX, heme *b*) containing electron transporter proteins, strict covalently to cystein (Cys) on motif of (-Cys-Xaa-Yaa-Cys-His-) [27]. HCOs of the type called *cbb*₃ were first found in nitrogen-fixing and pathogenic bacteria, where they are expressed under low oxygen tension and have a lower *K_m* for O₂ than A-type HCOs. More recently, members of the *cbb*₃ family not belonging to proteobacteria were reported [28, 29] weakening the evidence for a modern origin of the enzyme [13].

Type C oxidases (*cbb*₃ enzymes) have been found in only two bacterial groups - purple bacteria and CFB group [28]; Flexibacter, Bacteroides and Cytophaga (*R. marinus*). The purple bacteria is subdivided into five subgroups

from alpha to epsilon by defined on the basis of 16S r-RNA analysis. The alpha division includes species of the genera *Rhodospirillum*, *Rhodopila*, *Rhodopseudomonas*, *Rhodomicrobium* and *Rhodobacter* [30]. The *Rhodobacteraceae* family, the purple non-sulfur bacteria, is being a member of the alpha subgroup and the most diverse group of the phototrophic purple bacteria. The *cbb₃* oxidases have an essential role in the specialized energy metabolism that allows proteobacteria to colonize in microaerobic environments because of their higher oxygen affinity [4, 29]. For instance, *cbb₃*-type enzyme is the only energy-conserving terminal oxidase and the only O₂-reducing HCO whose sequence is represented in the genomes of some important human pathogens, *Helicobacter pylori*, which is the major cause of gastric ulcers, making the *cbb₃* oxidase a potential drug target and *Campylobacter jejuni* [8, 29, 31], also *Neisseria meningitides* [32] or *Brucella suis* [33], *Vibrio cholera* [34]. Hence, this family consists symbionts of plants (e.g. *Rhizobium* spp.) and animals, and a group of pathogens, the Rickettsiaceae. Moreover the precursors of the mitochondria of eukaryotic cells are thought to have originated from *Rickettsia* spp. in this class (endosymbiotic theory). Cytochrome *c* oxidase complexes are also found in a number of bacteria like, *Azorhobium caulinodans* [32], *B. japonicum*, *P. denitrificans*, *R. sphaeroides*, and *R. capsulatus* [35] that must have arisen within a different evolutionary branch than that which gave rise to the mitochondrial cytochrome *c* oxidase complex [32]. The facultative photosynthetic bacterium *Rhodobacter capsulatus* and its close relative *Rhodobacter sphaeroides* demonstrate substantial metabolic plasticity through its ability to grow in response to a variety of different environmental conditions (Fig. 1.2) with its highly branched electron pathways. For example, while growing

anaerobically in light, *R. capsulatus* utilizes a photosystem to generate energy. However, when growing in the presence of oxygen, the cells repress synthesis of the photosystem and instead obtain energy by aerobic respiration using two cytochrome oxidases, cytochrome *cbb₃* and ubiquinol oxidase, as terminal electron acceptors. Both oxidases reduce oxygen to water in an effort to remove excess reducing power concomitant with proton translocation. The generated proton potential is utilized for multiple cellular processes, including ATP synthesis via ATPase.

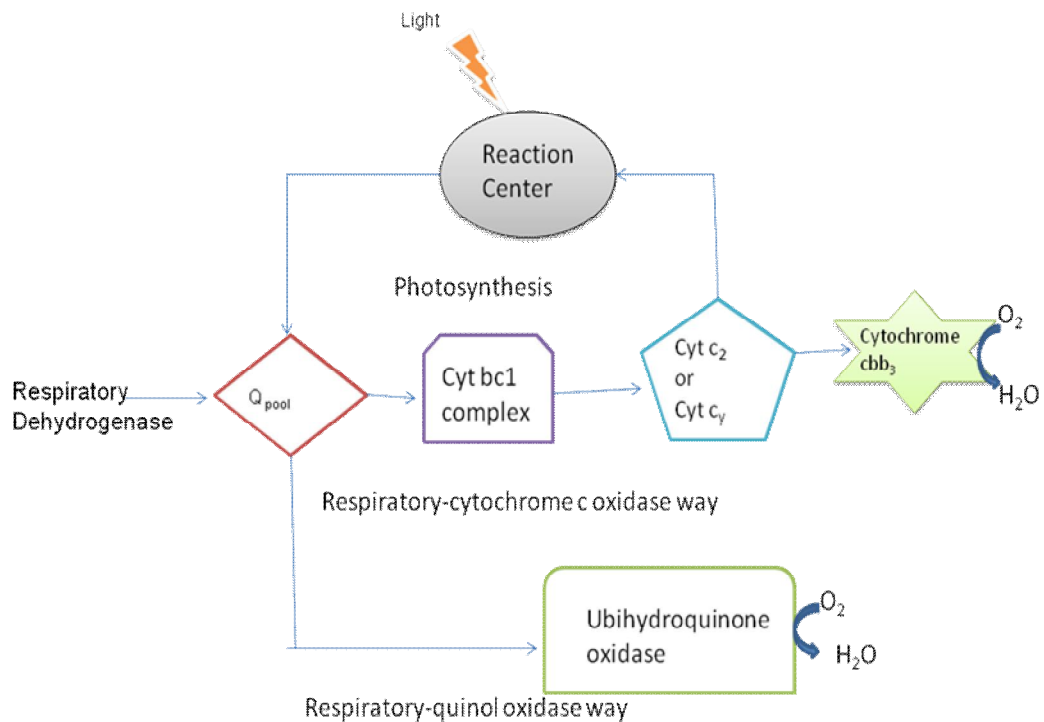


Figure 1. 2 The aerobic and photosynthetic electron transfer (ET) pathways in *R. capsulatus*. The electron flow circulation at photosynthetic ET pathway: Electrons come from reaction center which is activated with photon, and conduct successively ubiquinone pool (Q_{pool}), cytochrome *bc₁* complex and electron carrier *c*- type cytochrome proteins (cytochrome *c₂* or cytochrome *c_y*) and return back to the reaction center again. At respiratory ET pathway, electrons come from the different respiratory dehydrogenases (NADH, succinate and malate dehydrogenases) and conduct to Q_{pool}. After this step, electrons chose a way: cytochrome *bc₁* complex, cytochrome *c₂* or cytochrome *c_y* and cytochrome *cbb₃* oxidase enzymes are contained in mitochondrial ET pathway. Another respiratory pathway doesn't contain *c*-type cytochrome, contains ubiquinol oxidase (QH₂) [Modified from (38)].

The cyt *c* oxidase of *R. capsulatus* has been purified previously and characterized as being a novel *cbb*₃-type cyt *c* oxidase without a Cu_A center [36]. *Rhodobacter capsulatus* is a member of the Rhodospirallaceae family and it belongs to the genus *Rhodobacter*. *Rhodobacter capsulatus* is a gram negative, purple non-sulfur and facultative phototrophic soil bacterium (Fig. 1.3). Proteobacteria are phylogenetically divided into the so-called α -, γ -, β -, δ -, and ϵ -subgroups. Its light-driven photosynthetic electron transfer pathway contains a photochemical reaction center (RC): ubihydroquinone, cytochrome *c* (cyt *c*) oxidoreductase (cyt *bc*₁ complex) and a *c* type cytochrome as a mobile electron carrier. On the other hand, the respiratory electron transfer pathways of *R. capsulatus* are branched after the quinone pool and contain two different terminal oxidases, previously called cyt *b*410 (cyt *c* oxidase) and cyt *b*260 (quinol oxidase) [37]. There is a suggestion about quinol oxidases were proposed to evolve from cytochrome oxidases by loss of the Cu_A centre. Because authors claimed that in cytochrome *c* and quinol oxidases, proton translocation is not achieved by the same mechanism; for cytochrome *c* oxidases they proposed a proton pumping mechanism in which Cu_A would be involved, while for quinol oxidases a Q-cycle should be operative [4]. The pronounced metabolic versatility, including the ability to grow under dark, anaerobic conditions, makes these purple non-sulfur bacteria excellent model organisms for studying microbial energy transduction [37]. On the other hand, alternate respiratory pathway exists in *R. capsulatus*. It is independent of all *c*-type cyts and uses the quinol oxidase that is thought to contain cyts *b* and *d*. *R. capsulatus* can use various compounds such as dimethyl sulfoxide, triethylamine oxide or nitrous oxide as terminal electron acceptors to grown under anaerobic dark conditions. So, for *R. capsulatus* having the

metabolic versatility is a big advantage. The presence of functional *c*-type cytochromes can be detectable by NADI staining, phenotypically [38]. *R. capsulatus* has photosynthetic membrane and membranes seem as vesicles, bacteriochlorophyll and chromatophore, known as photon-collection pigment containing of cell [27].



Figure 1. 3 The electron microscopy photo's of *Rhodobacter capsulatus*.

The *cbb₃*-type oxidase has been identified as a heme-copper oxidase that catalyzes the reduction of O₂ to water and has a very high affinity for O₂ [29]. As a consequence, it allows respiration under low-O₂ (microoxic) conditions, such as those encountered by rhizobia in the legume nodule (O₂). Genes encoding the *cbb₃* oxidase complex were isolated initially from rhizobial species (*Bradyrhizobium japonicum*) and named *fixNOQP* for their role in symbiotic nitrogen fixation with host plant [29]. Since then, orthologous genes have been identified in other gram-negative bacteria and called *ccoNOQP* in non-N₂-fixing organisms [28]. Subsequently, the *fixNOQP* (*ccoNOQP*) operon has been identified in other proteobacteria, where it is always close to a second gene cluster, *fixGHIS* (*ccoGHIS*), whose expression is required for the assembly of a functional *cbb₃* oxidase [29]. These genes encode membrane-bound proteins which play a crucial role in enzyme maturation [13, 27]. These enzymes are encoded by four open reading frames with the common order *ccoN-ccoO-ccoQ-*

ccoP [13,39]. Analysis of the cluster of structural genes surrounding *ccoN* shows that the canonical organization of the structural operon is conserved in all members of the subgroups for which the phylogenetic positioning of the *ccoN* tree corresponds to that of a standard 16S rRNA tree.

CcoNOQP has multiple roles. It functions not only as a terminal oxidase but also as a redox sensor in a signal transduction pathway controlling photosynthesis gene expression [29]. The regulation of *ccoNOQP* expression has been investigated in many different bacteria. The recurrent finding is that O₂ constitutes a key regulatory element in controlling the expression of these genes. Not surprisingly then, O₂ is the primary effector of *ccoNOQP* expression. The O₂⁻ sensitive regulator Fnr regulates *ccoNOQP* expression in *Rhodobacter sphaeroides*, *Paracoccus denitrificans*, *Pseudomonas aeruginosa* PAO1, *Rhizobium leguminosarum* bv.viciae UMP791, *Rhodobacter capsulatus* MT1131 [2, 28]. This transcriptional regulator was first identified in *Escherichia coli*, where it controls the switch from aerobic to anaerobic respiration. Fnr proteins include an O₂-sensitive [4Fe-4S] cluster at their amino terminal part. Under low-O₂ concentrations, the dimeric form of these two proteins may play a role in the uptake and metabolism of copper required for the assembly of the dinuclear centre of cytochrome *cbb*₃ oxidase [28,40]. The regulatory systems regulate specific genes but also to integrate diverse metabolic activities and, thereby, to establish a hierarchy of gene expression [27]. The structural genes of this enzyme (*ccoNOQP*) have been sequenced in last decade from *R. capsulatus* 37b4 and aligned to the partial amino acid sequence of the purified enzyme from *R. capsulatus* MT1131 [36]. The biogenesis of a multisubunit protein complex containing several prosthetic groups, such as cyt *cbb*₃ oxidase, is likely to require

many accessory proteins involved in various posttranslational events, including protein translocation, assembly, cofactor insertion, and maturation in contrast to the other related species, an ORF containing a gene coding for a possible Fnr-like protein is absent upstream of *ccoNOQP* in *R. capsulatus*. Interestingly, no Fnr binding site is present in the *ccoP-ccoG* intergenic region of *R. capsulatus* [38]. Different oxygen affinities may be regulated by turn on or off cytochrome *c* oxidases by many facultative aerobic microorganisms.

A separate subfamily is formed by the *cbb₃*-type oxidases, defined by a unique subunit composition, three C-type hemes in the noncatalytic subunits, two B-type hemes in the central subunit, and the transfer of the catalytic tyrosine from helix 6 to helix 7 [18]. It contains at least a membrane-integral *b*-type cyt in subunit I [CcoN] with a low-spin heme *b* and a high-spin heme *b₃*-Cu_B binuclear center, and two membrane-anchored *c*-type cyts (CcoO and CcoP). Cytochrome *cbb₃* oxidases show very high affinity for its substrate oxygen by its active site, consist of a *b*-type heme, heme *b₃*, magnetically coupled to Cu_B. Cytochrome oxidases have different type of hemes; A-, B-, C-, and O-type (Fig.1.4).

Three different types of heme-copper oxidases

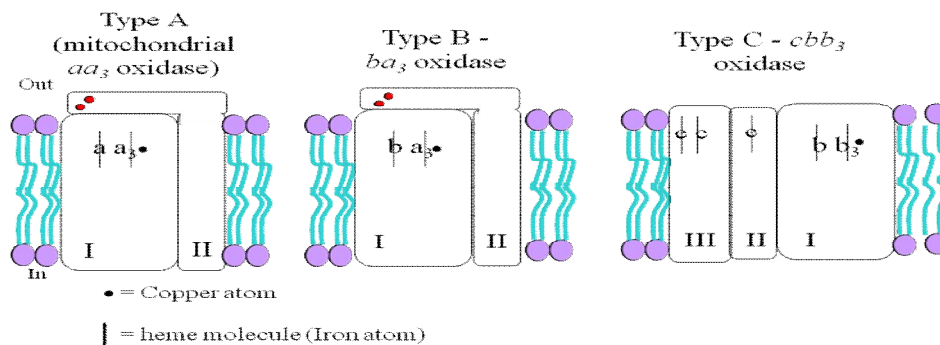


Figure 1. 4 Types of heme-copper oxidases

Due to *cbb₃*-type oxidases ability to reduce both O₂ and NO, it is thought that they are among the first oxidases to evolve, although this view has recently

been challenged. Subunits N and O are homologous to the NO-reductase subunits NorB and NorC, whilst subunits P and Q are unique for the *cbb₃*-family. Interestingly, the tyrosine of the Cu_B center, which has analogous to the *aa₃*-type oxidases, been shown to form a crosslink to the histidine ligand of the copper, is located in a different helix than the histidine. In *aa₃*-type oxidases these residues are both located in helix 7. It has been suggested that the tyrosine is in a different orientation compared to the binuclear site in the *cbb₃* oxidases compared to the *aa₃*-type oxidases, which might explain the differences in oxygen binding affinity between the two oxidases [1].

In contrast to the eukaryotic oxidases which express only *aa₃*-type oxidase, the bacterial enzymes can switch between different type of oxidase expression depending on external conditions [1]. The homology between *cbb₃*- and *aa₃*-type oxidases is low (about 14% sequence identity) and the residues constituting the D-pathway (see above) are missing in *cbb₃s* [16]. The *aa₃*-type cytochrome *c* oxidase predominates when the cells are grown aerobically with high O₂ tension. The alternate cytochrome *c* oxidase is the *cbb₃*-type oxidase, which is present under microaerophilic (low O₂) conditions and when the cells are grown photosynthetically [5]. There are similarities in the three-dimensional structures of the *cbb₃*-type cytochrome *c* within the whole heme-copper oxidase family. One of the main characteristics for all members of the heme-copper family, in addition to the redox center composition, is the presence of strictly conserved histidine ligands to the redox centers [41]. The other characteristic is the presence of three core subunits [8, 42].

It has been shown that the *cbb₃*-type oxidases play important roles in different environmental conditions such as low-oxygen growth conditions. In

Bradyrhizobium japonicum, the *cbb₃*-type oxidase is essential for nitrogen fixation. In these bacteria this oxidase is expressed under microaerophilic growth conditions and shows high affinity for oxygen. Additionally, *cbb₃*-type oxidases can also play important roles in the aerobic growth, as in *Rhodobacter capsulatus*, where cytochrome *cbb₃* is the sole cytochrome *c* oxidase present [39].

The *cbb₃*-type oxidase is made up of three to four subunits; subunit I is encoded by *ccoN* and is a membrane-integral *b*-type cytochrome with a high-spin heme-Cu_B binuclear center and a low-spin heme. Subunits II and III are encoded by *ccoO* and *ccoP*, respectively, and are membrane-anchored *c*-type cytochromes that transfer electrons from cytochrome *c₂* to subunit I. As shown in *Rhodobacter sphaeroides* and *Bradyrhizobium japonicum*, the *ccoN*, *ccoO* and *ccoP* gene products are essential for both the activity and the assembly of the *cbb₃* oxidase. In contrast, *ccoQ*, which encodes subunit IV, is sometimes missing. The *ccoQ* is not required either for the assembly or activity of the oxidase under low-O₂ concentration but may play a protective, stabilizing role under oxic conditions, especially for CcoP which is very susceptible toward proteolytic degradation [43]. Hence, high availability of O₂ may post-transcriptionally lower *cbb₃* oxidase abundance and the activity of those enzymes lacking subunit IV [28].

The quinol oxidase exists in aerobic conditions [15]. Cytochrome *c* oxidase is a redox-driven proton pump. In *P.denitrificans* and *R. sphaeroides*, they pump one proton per electron. The oxidases separate charges across the membrane. The eight protons cross the membrane per O₂ and one proton is pumped for each electron used in oxygen reduction chemistry. Overall reaction is as follows:



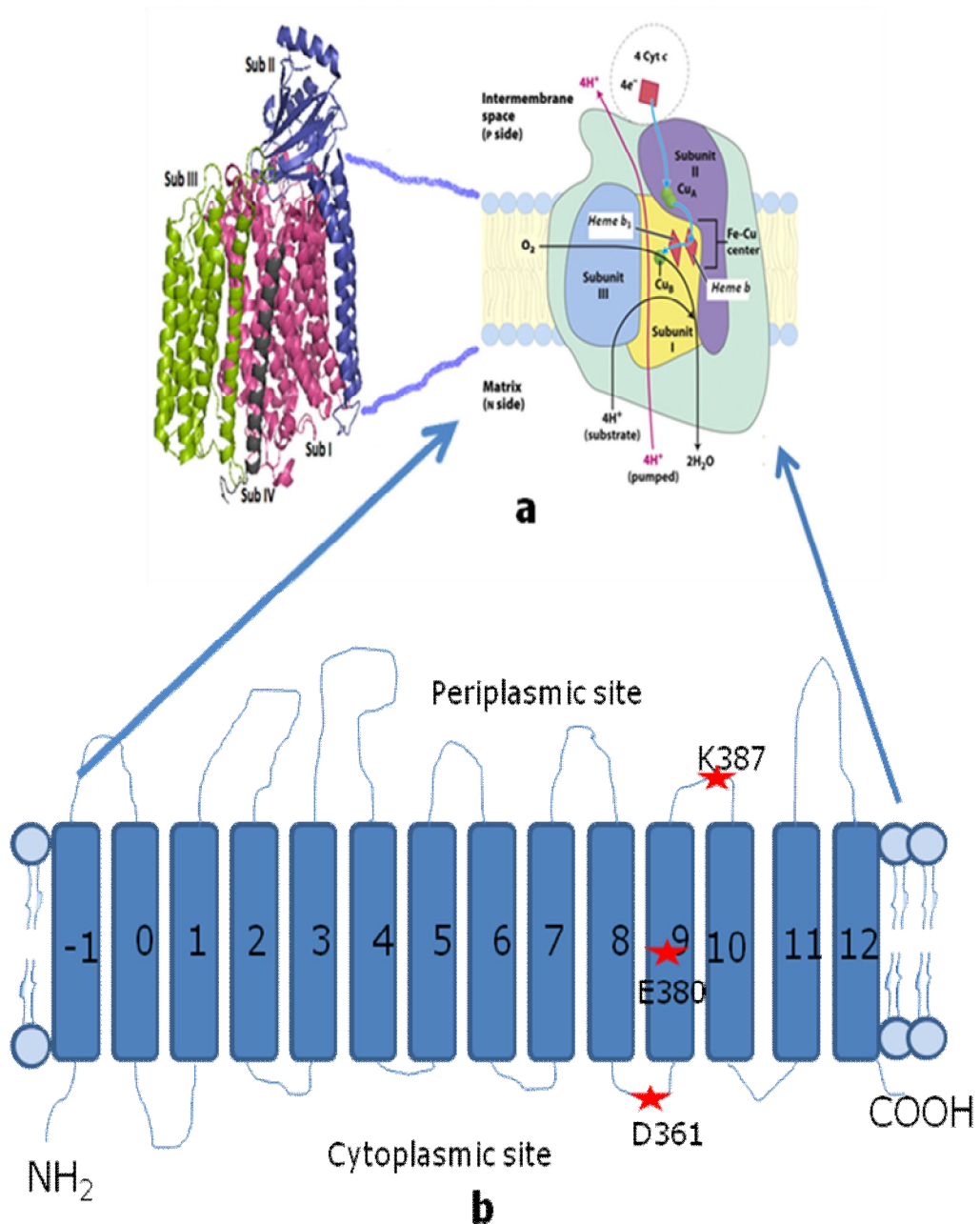


Figure 1. 5 (A) Organization of the *ccoNOQP* subunits of *cbb*₃-type oxidase in *R. capsulatus*. The *ccoNOQP* operon of *R. capsulatus* encodes the four structural proteins that comprise the cytochrome *cbb*₃ oxidase complex (Modified from [16]). (B) Topological model of subunit I (*ccoN*) of *cbb*₃-type oxidase in *R. capsulatus*. Stars on helix represent conserved amino acids in subunit. The stars in cytoplasm, on the helix nine and periplasm represent: D361, E380 and K387, respectively (Modified from [44]).

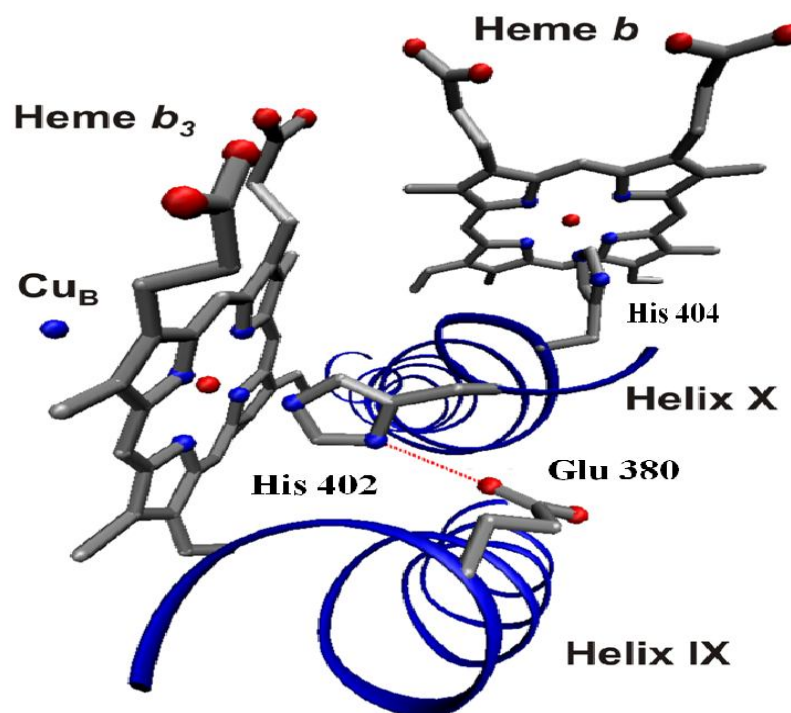


Figure 1. 6 A model of the active site of cytochrome *cbb₃*. The hemes and the copper center of the catalytic subunit are shown together with the axial histidine ligands of the hemes originating from helix X. The glutamate 380 residue suggested to form a hydrogen bond (dotted red line) with histidine 402 is located in helix IX. The figure was prepared using the Visual Molecular Dynamics software (modified from [45]).

1.2.4. The Structure and Prosthetic Groups of Subunit I

Subunit I is the core of the multisubunit complex and defines the heme-copper superfamily, additionally best conserved among the HCOs [3, 4, 19]. The first gene of the *ccoN* encodes a basic membrane protein of 535 amino acid residues, with a predicted molecular mass of 45 [38, 39]. It shows a highly homologous to *fixN* gene product of *B. japonicum*, *R. meliloti* and *A. caulinodans* (62-77% positional amino acid identity and 79-84% similarity) [38]. Hydropathy analysis shows 14 transmembrane segments (Fig. 1.5-b) with both N and C termini facing the cytoplasmic side and conserved histidines take place in this subunit [39]. Hence, there are two different helices of CcoN. The high degree of

hydrophobicity for the first two helices (helices -1 and 0) might not have a role in the function/structure of the oxidase since they are missing in the *Helicobacter pylori* CcoN sequence, in which the predicted protein starts at the level of the third transmembrane segment of the rest of the sequences [9]. When viewed from the periplasmic side, the 12 transmembrane α -helical segments which form three semi-circles consisting of four helices each. These centers are located positive (outer or periplasmic) and negative (inner or cytoplasmic) membrane sides, respectively [4]. These make protein scaffold for the redox center and provide the three pores (Fig. 1.7), designated, A, B and C are formed in the center of these semi-circles [3]. Helices XI, XII, I and II form the first arc; pore C, III- IV the second; pore A, and VII to X the third; pore B. These "pores" are blocked; pore A by preferentially conserved aromatic residues, pore B by high-spin heme; b_3 and Cu_B , and pore C by low-spin heme; heme b . The high-spin's propionate groups are pointing towards the intermembrane space in mitochondria and periplasmic space in bacteria. The hydroxyethylfarnesyl side chain penetrates into the lipid bilayer [3]. Side chains of the aromatic amino acid residues may participate in long-range electron transfer between the metal centers or provide a binding pocket for the prosthetic groups. Alternatively, aromatic amino acid residues may provide a tightly packed hydrophobic environment for the buried portion of hemes [46]. The high-spin heme forms the binuclear, oxygen-reducing site together with a copper ion, Cu_B . In the interface region between subunit I and II, in addition to the redox centers, a non-redox-active metal can be assigned: $\text{Mg}^{2+}/\text{Mn}^{2+}$ and $\text{Ca}^{2+}/\text{Na}^+$ [in different species different metal ions occupy the binding sites] [7, 8]. The importance of this metal site is not known. It has been suggested to have a structural role and/or it may coordinate the interaction of

subunits I and II [3, 9]. Although the function of the metal center is not completely clear, but may be related to enzyme stability, regulation and ejection of protons to the P-side of the membrane. Mutations targeting the Ca^{2+} ligands have revealed that the metal is not necessary for catalytic activity and proton pumping [7,47]. In resulting mutant enzymes, where the metal site was empty, proton translocation was unaffected and the catalytic activity was only slightly inhibited. This site is not present in the quinol oxidases. Therefore, a possible regulatory role for this metal ion in the electron transfer from Cu_A to heme a could be conceivable [3]. The metals of all three redox centers are located at about the same depth within the membrane: $\sim 1/3$ of the way from the P-side and $2/3$ from the N-side [8].

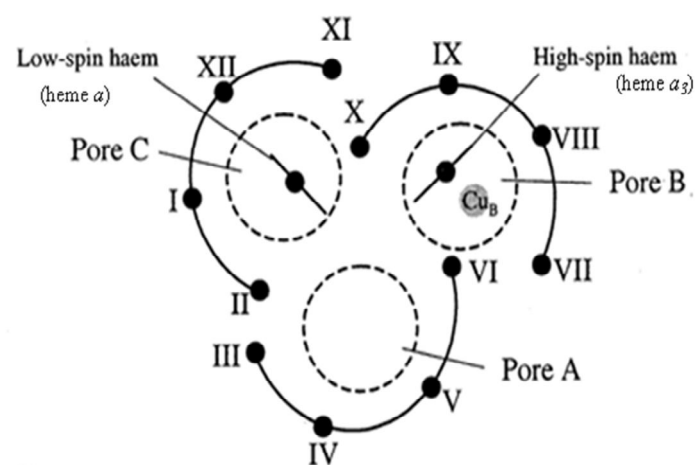


Figure 1. 7 Schematic view of subunit I of the cytochrome c enzyme observed from the negative site of the membrane (modified from [5, 22]).

Only subunit I is common to all HCOs and as already pointed out, subunit I contains the ligands for two hemes and Cu_B in catalytic center [4, 5, 19]. The Cu_B site of CcO is a unique center for heme-copper oxidases, and has so far not been found in any other protein, although the related NO-reductases have a Fe_B center, probably with a similar structure to the Cu_B -center. The copper atom of

Cu_B is located 4-5 Å from the iron atom of heme *a*₃ [1]. First one of hemes is called high-spin heme (heme *a*₃). This heme provides oxygen-binding site because of it is part of the bimetallic catalytic site and coordinated to a single histidine; [His-402] [4, 5, 19]. The other component of the bimetallic active site is a copper atom, Cu_B which is coordinated with three histidine ligands; H264, H314 and H315 are probably ligands of Cu_B [38]. Whereas the binuclear center is in pore B as mentioned previous [19]. The low spin-heme (heme *a*) is located in pore C, and it facilitates the transfer of electrons from Cu_A to the catalytic binuclear center. This heme is ligated to two histidines; H114, H404. The axial ligands of the iron, Fe_a, are His94 (His61) and His413 (His378) from transmembrane helices II and X, respectively with its propionate groups pointing towards the intermembrane space in mitochondria and periplasm in bacteria. The hydroxyethylfarnesyl group points towards the matrix in mitochondria and cytosol in bacteria. The heme plane is perpendicular to the membrane and the iron is located at a depth of about 1/3 of the membrane thickness from the outside [3, numbering from *P.denitrificans*].

Table 1. 1 Some key residues in cytochrome *c* oxidases

Conserved Amino Acids in the Heme-Copper Oxidase Superfamily			
type or location	<i>B. Taurus</i> (<i>aa</i> ₃)	<i>T. thermophilus</i> (<i>ba</i> ₃)	<i>R. capsulatus</i> (<i>cbb</i> ₃)
invariant metal ligands			
low-spin heme Fe	H61, H378	H72, H386	H114, H404
high-spin heme Fe	H376	H384	H402
Cu _B	H240, H290, H291	H233, H282, H283	H264, H314, H315
active site			
loop3-4	Y129	Y136	Y178
loop11-12	R439	R450	R468
helix 6	W236	W229	W260
helix 6	V243	V236	V267
other conserved residues			
helix 8	P315	P308	P339

helix 9	G355	G363	G381
helix 11	G420	G431	G448
helix 11	G432	G443	G460
subfamily-specific functionally important residues			
D-channel	D91, E242	-	-
K-channel	K319	-	H300 ^a
His-Tyr	Y244	Y237	
^a Forming an analogous channel			

*modified from [40]

The three types of heme (Fig. 1.8) associated with subunit I of the heme-copper oxidases; A, B and O. Individual oxidases have different combinations of these hemes. Heme O differs from heme B by the addition of a hydroxyethylfarnesyl side chain. Heme A has a formyl group instead of methyl group which is presented in heme B and heme O [15]. Heme *a* as well as heme *a*₃ are type A hemes which are characterized by being non-covalently bound to the protein [47]. For historical reasons, the subscript 3 is used to denote the O₂-binding (and CO-binding) high-spin heme, so this enzyme is referred to as cytochrome *aa*₃, or an *aa*₃-type oxidase. However, if heme B is in the low-spin site, as is the case for the cytochrome *c* oxidase from *T. thermophilus*, the resulting oxidase is called a *ba*₃-type oxidase [5]. On the other hand, classify the oxidase family using the types of heme can not be true without thinking their types of electron donor and amino acid sequence similarities [4]. Hence, the residues binding these prosthetic groups (six histidines) are strictly conserved in all heme-copper oxidases and are highly diagnostic of the family [2, 4, 41]. However, a novel cross-linked histidine-tyrosine cofactor in the active site have been identified [19]. It has been postulated that this histidine-tyrosine covalent bond may alter the chemical properties of the tyrosine in such a way that it can participate in the catalytic cycle as donor of a proton and an electron, thereby forming a tyrosine radical [3].

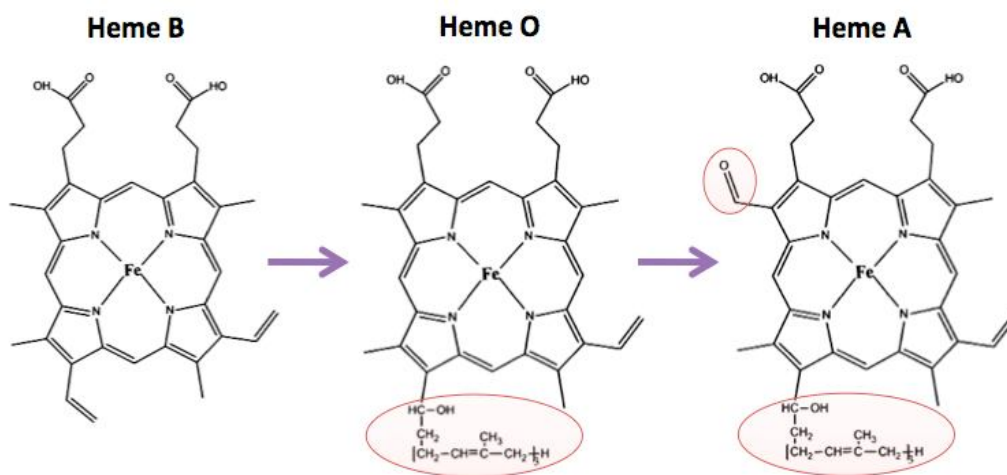


Figure 1. 8 Three types of hemes, heme B, heme O, and heme A [16].

The electronic coupling between heme *a* and the binuclear center is very strong, and fast electron transfer between these two redox centers has recently been used to explain the high apparent oxygen affinity of oxidase by kinetic trapping of bound oxygen [47].

The variation in heme-copper oxygen reductases is due to the differences in the active site and proton channels. The A- and B-families need either an *o* or *a* heme in the active site (the hydroxyl group on the tail is part of the K-channel), whereas the C-family utilizes *b* heme in the active site (they use a conserved tyrosine in place of the tail) [15].

1.2.5. The Structure and Metal Center of Subunit II

Subunit II consists of two transmembrane helices, an N-terminal loop and a C-terminal globular domain with an overall mass of ~27 kDa and encodes *ccoO* gene as a 242 amino acid polypeptide [8, 39, 47]. It locates above pore B of subunit I [47]. Subunit II brings electrons to subunit I and contains a second copper-containing redox center called Cu_A [5]. It is composed of a transmembrane

helical domain and peripheral domain, in the positive (outer or periplasmic) site of the membrane. In these cytochrome *c* oxidases, the binding site for cytochrome *c* has been localized in subunit II and Cu_A serves as an initial electron acceptor from reduced cytochrome to funnel electrons into the “catalytic core” of the enzyme. Hence, despite of the major role of subunit II may provide the substrate binding site, either for quinol or cytochrome *c*, quinol oxidases lack the Cu_A redox center [7]. In all known cytochrome oxidases, this centre is binuclear, which has been explained by the unusually low electron transfer reorganisation energy at this site, such that electron transfer is not the rate-limiting step [4]. Cu_A is present only in the cytochrome *c* oxidase sub-family and is absent in the quinol and *cbb*₃ type oxidases [8]. Subunit II consists of two alpha helices and a hydrophilic anti-parallel betasheet. The Cu_A binding site was originally assigned as a mononuclear copper site but later found to be a mixed-valence binuclear site [3]. Hydropathy analysis shows that CcoO probably has a single membrane-spanning domain near the N terminus, and a hydrophilic domain, which is most probably exposed to the periplasmic side where heme C is attached [39].

1.2.6. Subunit III

Subunit III binds to subunit I on the side opposite to subunit II without any contact to it and has a mass of ~6.5 kDa [8]. The *ccoQ* gene codes for a 58 amino acid acidic polypeptide [34, 38, 39]. It consists seven transmembrane helices without any redox active centers (no redox cofactors and has almost no extramembrane part) [7, 8] and these helices arranged into two bundles. One bundle is formed by the first two helices and the other by the last five helices [3].

The association of subunit III with subunit I is weak [47]. In the absence of subunit III, the O₂ reduction activity of the enzyme slow down [7, 8], seems not required either for the assembly or activity of the oxidase under low-O₂ concentration. However, based on gene deletion studies it was proposed that subunit III is needed for the correct assembly of the enzyme and it may also stabilize the mature oxidase, or in activity regulation, and protective role under oxic conditions [3, 28, 39]. Moreover, the role of subunit III might be to prevent blockage of the oxygen channel entrance by other proteins. The structure of the bacterial oxidase indicates a possible channel in the interface of subunit III for diffusion of oxygen leading from the cleft of subunit III to the binuclear center [1, 47]. It has a high hydrophobic amino acid stretch near the N terminus, and a charged motif at the C terminus as previously suggested [38, 39]. Hence, CcoQ subunit serves as a protector of *cbb₃* oxidase from oxidative destabilization in the presence of oxygen by lacking CcoQ to inactivate the enzyme [34]. Hence, high availability of O₂ may post-transcriptionally lower *cbb₃* oxidase abundance and the activity of those enzymes lacking subunit IV [28]. Recently, there are some suggestion about CcoQ serves as a transporter in signal transduction pathway that controls the expression of photosynthesis-related genes in *R. sphaeroides* and it makes this by presence of two histidine residue that are conserved in *R. sphaeroides* and *R. capsulatus*, but which are not present in non-photosynthetic species [48, 49].

1.2.7. Subunit IV

Subunit IV has one transmembrane helix with the N-terminus on the cytoplasmic side and is not homologous to any previously known protein [3, 7, 47]. The last gene, *ccoP*, codes for an acidic membrane protein of 297 amino acids and calculated Mr of 32 kDa [38, 39]. Its function remains a mystery, especially since it bears no significant homology with any other known peptide or protein. Deletion of the gene encoding subunit IV has no effect on enzyme expression and activity [7]. Some evidences suggest that this subunit is important for stability of Cu_B center in subunit I [47]. Hydropathy profiles predict two main domains; a hydrophobic transmembrane domain near to the N terminus, and a hydrophilic domain most probably facing the periplasmic side [38, 39]. The eukaryotic enzymes have ten additional nuclear encoded subunits, of which seven are single transmembrane-helices. The function of the additional subunits is possibly regulatory, and might increase the enzyme stability [1]. A number of lines of circumstantial evidence suggest that CcoP might serve as a gas-sensing rather than electron-receiving domain [32].

1.2.8. Mechanisms for Proton Translocation by Cytochrome *c* Oxidase

As discussed above, the energy released during the catalytic cycle of the enzyme is transduced to an electrochemical proton gradient across the membrane by two means. First, the chemical reaction directly generates electrical potential as electrons and protons for dioxygen reduction are obtained from the opposite sides of the membrane. Secondly, the enzyme is a proton pump which translocates one proton across the membrane per each electron transported to

dioxygen. During catalytic cycle electrons followed sequential transfer from reduced cytochrome *c* to Cu_A, heme *a*, and finally to catalytic site (heme *a*-Cu_B). Because in each catalytic cycle four electrons are needed to reduce one O₂ molecule, a number of distinct steps (Fig. 1.9) can be identified in the reaction.

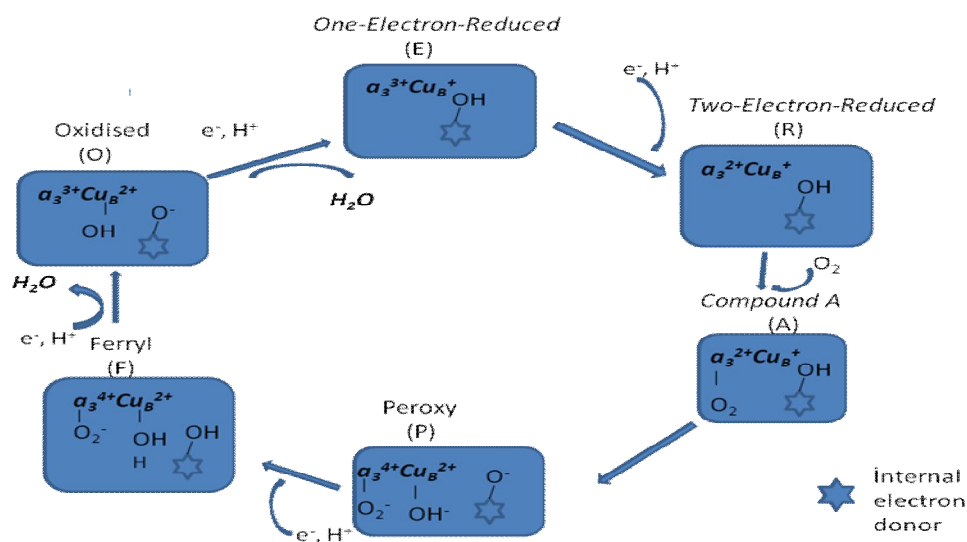


Figure 1. 9 Catalytic cycle (Modified from [16]).

1.2.9. Pathways

The CcO catalysis requires delivery of three substrates – electrons, protons, and O₂ – to the binuclear center, and expulsion of H₂O molecules produced in the binuclear center. Each substrate or the product takes a specific route through the enzyme. Electron transfer occurs without a specific structure to move through the protein, but the transportation of each of the other substrate/product does require its own channel.

1.2.9.1. Electron Pathways

The basic model of electron transfer between two sites was developed by Marcus [50]. The theory states that the speed of electron transfer between two redox centers depends on three factors: [i] the difference in E_{ms} (midpoint redox potential) between the two centers, [ii] the distance separating them, and [iii] the reorganization energy, i.e. the energy required to alter the structures of the centers' environment in response to the electron transfer [8]. The reduction of O_2 to H_2O by CcO requires four electrons that are provided one by one by cytochrome *c*. Cytochrome *c* docks to CcO at the interface between subunits II and III. The docking is accomplished via the electrostatic and, perhaps, also the hydrophobic interactions. The first e^- acceptor in CcO from cytochrome *c* is Cu_A . Subsequently, the e^- is transferred to heme *a*. Although the distances [metal-to-metal] from Cu_A to heme *a* and heme a_3 are comparable, the electron transfer occurs between Cu_A and heme *a*. Then, the e^- is transferred from heme *a* and heme a_3 . However, an initial partial nanosecond electron transfer from heme *a* to heme a_3 is present that is not coupled to the H^+ translocation. The final step of the electron transfer events is from heme a_3 to Cu_B which occurs on a time scale of hundreds of microseconds due to its coupling to the proton transfer.

1.2.9.2. Exit Paths for Protons, Oxygen and Water Molecules

The domain on the external side of the binuclear site is highly hydrophilic containing several charged and polar amino acid residues, the propionates of the two hemes, a Mn^{2+} or Mg^{2+} and several water molecules. The hydrogen bonded network they form could participate in the pathway by which translocated protons

exit from the enzyme structure [3, 38]. According to some authors, this pathway serves as an exit for the water produced, but some says, preferred a role in the pathway of pumped protons from the dinuclear centre to the outside. This possible water channel, which is lined with hydrophilic residues proceeds from the external side of heme a_3 along the subunit I-II interface to the outside surface of the enzyme [3]. Studies have shown that the water produced in the binuclear center (BNC) takes a specific route that passes next to the Mg^{2+}/Mn^{2+} binding site on their way to the P-side of the membrane [7, 8]. The exact exit path of the water molecules is not resolved, but it is presumably located at the interface between subunit I and II [7, 38]. As the maximum turnover of CcoO is *ca.* 1000 e⁻/s, 500 water molecules are produced every second in bacterial enzymes. This leads the water molecules to the Mg^{2+}/Mn^{2+} center [1]. The water channel suggested in the mammalian X-ray structure is similar to the hydrophilic cleft observed in the bacterial enzyme proceeding from the binuclear center to the outside of the membrane [9].

Along with the necessity of getting water and protons out of the active site, oxygen must be brought in. Non-polar molecules such as oxygen can diffuse through lipid membranes. However, for oxygen to diffuse into the buried active center of CcO, a special oxygen diffusion channel has evolved. It has been shown by combined structural studies and mutational analysis that certain amino acids which are important in forming the channel [1]. The channel starts from the hydrophobic part of the membrane, where O₂ solubility is the highest. The proposed entrance point of the channels is located in either subunit II or III leading further to subunit I and to the BNC via a conserved valine residue Val-279, whose important role was confirmed by mutagenesis studies [8]. Since

oxygen is an order of magnitude more soluble in organic than aqueous solvents, entry of O₂ to the active site would be expected to take advantage of higher O₂ concentrations in the membrane and facilitate diffusion through hydrophobic regions of the protein. In fact, a “hole” in the space-filling model of the coordinates allows a residue close to Cu_B (E286, *R. sphaeroides*; 278, *P. denitrificans*; 242, bovine) to be seen from the surface. This channel would appear to have the advantage of a built-in oxygen reservoir. It could be argued, of course, that a facilitated oxygen channel is unnecessary, since oxygen concentrations are normally far in excess of the O₂ affinity of the enzyme, so access of O₂ would not be rate limiting. However, recent mutational efforts to block the putative O₂ channel from the subunit III pocket provide good support for a rate-limiting, favored access route [9].

1.2.9.3. Proton Input Pathways

The catalytic reaction performed by all terminal oxidases, i.e., the reduction of O₂ to H₂O ($\text{O}_2 + 4\text{e} + 4\text{H}^+ \rightarrow 2\text{H}_2\text{O}$) involves four protons. As the reaction site is well embedded in the protein, intra-protein channels for proton conduction must be always present in subunit I. In CcO, two proton-conducting pathways have been identified on the basis of results from site-directed mutagenesis experiments and from an inspection of the available crystal structures [51]. These proton channels were named D- and K-channel according to those conserved residues [4] and they were identified based on site-specific mutagenesis and by X-ray structures analysis [8]. These pathways connect the inner aqueous phase and the binuclear site [52].

The mechanism of the proton transfer is very different from that of electron transfer. While the latter most likely requires no specific pathway inside a protein, the transfer of protons occurs within channels filled in with water molecules. Proton translocation in these pathways has been proposed to occur via hydrogen-bonded chains, so called “proton wires” which include amino acid residues and bound water molecules [52]. The mechanism of the proton transfer along a water chain was first proposed by Grotthuss in 1806 [53]. Grotthuss stated that the proton transfer occurs without transportation of mass of a H^+ ; the proton transferred stepwise between neighbouring water molecules [4, 52]. In a protein, H^+ pathways are made up of lines of H_2O molecules that are stabilized by their hydrogen-bonding with the surrounding polar amino acids of the protein. The O_2 reduction chemistry requires four protons to be delivered to the BNC for each O_2 molecule [= ‘chemical’ protons]; four more protons are translocated across the membrane [= ‘pumped’ protons]. All eight protons are taken from the N-side of the membrane. The proton transfer in CcO is coupled to the electron transfer and is driven by the energy of the redox drop of electron transfer from cytochrome *c* to O_2 . The mechanism of the H^+ translocation is therefore called the *redox-linked proton pumping*. Initially, the D- and K-channels were proposed to separately supply the ‘pumped’ and ‘chemical’ protons, respectively. However, later experiments suggest, the individual proton is just relayed from the donor to the acceptor, followed by a conformational reorganization [52].

1.2.9.3.1. The K-Channel in Cytochrome *c* Oxidase

The K-channel is named after lysine (K362 in the *R. sphaeroides*) a conserved residue located in the middle of the pathway, at about half the distance

from the N-side to the BNC. It starts with a serine, (S299 in the *R. sphaeroides*) or a glutamate, (E101 in sub II in *E.coli*) and continues up to the BNC through K (362) [24]. The K-channel leads directly to the BNC being located in pore B [4]. The K-channel is on the bacterial cytoplasmic surface (mitochondrial matrix) of the enzyme and leads exclusively to the BNC [22]. It is shorter than the D-channel. K-pathway plays a role for proton uptake associated with first electron transfer to the enzyme [54]. Water molecules have also been predicted to participate in proton conduction in the K-channel [3]. Two to three water molecules have been resolved within the channel. The K-channel is responsible for the uptake of one or two ‘chemical’ protons in the reductive half of the catalytic cycle and for reduction of channel prior to the binding of oxygen by helping of K362 [22, 55].

1.2.9.3.3. The Structure and Function of D-Channel

One of the putative proton-transfer pathways leading from solution toward the binuclear center in many cytochrome *c* oxidases is the D-pathway, so-called because it starts with a highly conserved and functionally essential aspartate [D (132), *R. sphaeroides*] residue and the entrance of the D-pathway is located on the N-side of the membrane in the loop between helices II and III [3, 7]. It continues further towards the middle of membrane and it includes both polar amino acid residues and a string of water molecules, connecting the N-surface to a region near the heme–copper active site. D132 is a highly-conserved residue which has been proposed to act as a “proton antenna”, recruiting protons from the cytosol to be transferred through the D-channel. At the entry of the D-channel,

Asp-124 together with a series of His residues form a H^+ -collecting antenna that facilitates the H^+ uptake from the liquid state. The D-channel continues to the H^+ exit channel [8]. D pathway starts from D132, surface of the enzyme facing the bacteria cytoplasm (mitochondrial matrix) and extends to E286 (*R. sphaeroides*) in the middle of the protein, which is suggested to be the branch point for the substrate and pumped protons. In the D pathway around 10 water molecules are aligned and facilitate proton translocation [22]. The oxygen reduction chemistry steps, which take place in the catalytic site, are well studied but proton pumping mechanism at the molecular level or the proton exit pathway are not understood yet [15]. One of the amino acid residues that have been suggested to be involved in gating the proton flow through cytochrome *c* oxidase is glutamate 286 (E286), a highly conserved amino acid residue in the mitochondrial type oxidases, located in the so-called D-proton-transfer pathway, used for transfer of both substrate and pumped protons during enzyme turnover [56,57]. Glutamic acid-286 is located next to the binuclear center and critically responsible for proton pumping as a component and highly conserved residue of the D-channel [18].

This channel seems as two parts: hydrophilic one, starting at the surface of the molecule facing the negative side of the membrane and ending at GluI-278, and a hydrophobic one, leading from the glutamate to the binuclear centre [4]. Glu-278 is a branching point of the H^+ channel from where protons are either transferred to the catalytic site (chemical protons) or towards the heme propionates and eventually across the membrane (pumped protons) between the BNC and the 'pump site', becoming de-protonated in the process [7]. Then, it accepts H^+ from the D-channel and the protolytic cycle repeats [8]. The D-

pathway is filled with water molecules, all the way from the protein surface to Glu278, which can facilitate fast proton transfer through a Grotthuss-type of mechanism [7, 8, 57]. Based on these results, the Glu278 residue has been directly implicated in the oxygen reduction and proton pumping mechanism [3]. The individual proton is just relayed from the donor to the acceptor, followed by a conformational reorganization [52]. The D-channel is responsible for the translocation of six protons out of the eight transferred for each O₂ molecule reduction event: the two ‘chemical’ protons that are taken up in an oxidative part of the catalytic cycle, and all four ‘pumped’ protons [8]. It has been proposed recently that the D pathway is better suited to the rapid uptake of protons than the K pathway, because the entrance of the D pathway is surrounded by a proton-collecting antenna and because “the D pathway site is rapidly protonated,” whereas “protonation of the K pathway is a long continuous process” [58].

In *c* type oxidases (*cbb*₃) have conserved histidine ligands in their catalytic active sites as mentioned above and also contain important and highly conserved tyrosine residue which connects to histidines in catalytic sites. The three histidine ligands of Cu_B and region of the Cu_B ligand within helix VI is one of the best conserved domains and therefore it is predicted to be structurally very similar among all heme-copper oxidases [59]. On the other hand, this tyrosine residues are detected in A and B type cytochrome oxidases by sequence alignment but missing in all C type oxidases and has been suggested to have a key role in the O₂ reduction chemistry; the hydroxyl group of the tyrosine may donate both an electron and a proton to break the O–O bond [59]. Because the metal centers of the active site can provide only three electrons out of the four needed in the full

reduction of molecular oxygen to water. Recently the structural model predicts, the active-site tyrosine in the *cbb₃* oxidases is located in transmembrane helix VII, whereas the active-site tyrosine observed in the type A and B oxidases is located in transmembrane helix VI. Furthermore, the predicted active site tyrosine in the type C oxidases is completely conserved within the family of *cbb₃* oxidases [10].

In the present study, a sequence alignment of forty different strains' catalytic subunits (Fig. 1.10) indicated that glutamate 380, asparagine 361 and lysine 387 (*R. capsulatus* numbering) were highly conserved in *cbb₃* type cytochrome oxidases of IX helix. There are some studies especially on glutamic acid residue (E286 numbering of subunit I from cytochrome *bo₃* of *E. coli* , E383 for *R. sphaeroides*) as being a component of the D-channel by locating next to the binuclear center and also, it is responsible for proton pumping for heme-copper oxidases [18, 19, 40, 56, 60]. Therefore, E380 locates on helix IX of subunit I *cbb₃* oxidase of *R. capsulatus*. This glutamate is located on the other side of the D-channel of *R. sphearoides* enzyme (locates on helix VIII). Such structural substitutions of functionally important residues have been thought for the other residues. These amino acids are highly conserved in C type family members. Recent data suggested that E380 [18, 19, 40, 61], D361 [19, 40] and K387 must have critical roles on enzyme activities.

1.2.10. PCR Methods of Site-Directed Mutagenesis

Early work on the development of the PCR method of DNA amplification showed its potential for mutagenesis [62]. Single bases mismatched between the

amplification primers and the template become incorporated into the template sequence as a result of amplification. Site-directed mutagenesis methods are useful to design the desired mutation on a specific region of interest gene. In 1988, a variation of the basic method which enables a mutation in a PCR-produced DNA fragment to be introduced anywhere along its length. The advantage of a PCR-based mutagenic protocol is that the desired mutation is obtained with 100% efficiency by in vitro conditions. PCR based site-directed mutagenesis is used to amplify circular plasmid and to insert a mutation anywhere in the plasmid with no requirement for convenient restriction enzyme sites.

In vitro mutagenesis methods are very useful when working with small plasmids carrying cloned gene. In this technique, besides target and known DNA line, two primers are required to amplify initially. One primer contains one or a few mismatches which will generate the site-directed mutation. The first cycle of PCR generates linear plasmid molecules, from one or both circular template strands. Subsequent rounds of PCR further amplify the linear plasmid sequence generated by the first PCR cycle, which is then isolated, ligated and used for transformation. This method has the advantage of introducing mutations at any desired site regardless of restriction sites are present in the surrounding sequence.

In this work, target codons encoding the three conserved amino acids, D361, E380 and K387, of cytochrome *cbb₃* structural genes of *Rhodobacter capsulatus* were substituted for D361N, E380Q and K387M by using this technique to understand the effects on enzyme activity. After generation of

mutations in vitro mutagenesis technique, genes have been cloned in a broad host range plasmid, pOX15/HB101. Plasmid pBluescript II SK+ (Stratagene) was used as a template DNA for site-directed mutagenesis.

1.2.11. Conjugation (Triparental Mating)

Triparental mating is a form of bacterial conjugation where a conjugative plasmid (helper) present in one bacterial strain assists the transfer of a mobilizable plasmid present in a second bacterial strain (donor) into a third bacterial strain. Recipient plasmids are introduced into bacteria for such purposes as transformation, cloning, or transposon mutagenesis. Triparental matings can help overcome some of the barriers to efficient plasmid mobilization. For instance, if the conjugative plasmid and the mobilizable plasmid are members of the same incompatibility group they do not need to stably coexist in the second bacterial strain for the mobilizable plasmid to be transferred. So, a *helper strain*, carrying a conjugative plasmid that codes for genes required for conjugation and DNA transfer, is required as the second strain. There must be a *donor strain*, carrying a mobilizable plasmid that can utilize the transfer functions of the conjugative plasmid, and a *recipient strain*, you wish to introduce the mobilizable plasmid into. Most engineered plasmid systems utilize a mobilizable but not selftransmissible plasmid vector, with the transfer functions provided in *trans* either from a helper plasmid such as pRK2013, a derivative of pRK2, in a triparental mating [63].

	Helix VIII	Helix IX	
<i>Rhodobacter capsulatus</i>	: SCAMDKLR	TPVIRMMVVSIGFYGMSTFEGPMMSTRAVNSLSHY	308
<i>Rhodobacter sphaeroides</i>	: SCAMDKLR	TDPIIRMMVVSIGFYGMSTFEGPMMSTRAVNSLSHY	311
<i>Paracoccus denitrificans</i>	: SCAMDKLR	TDPIIRMMVAVGFYGMATFEGPMMSTRAVNSLSHY	315
<i>Azoarcus</i> sp.	: SCAMHKL	RDPILKFLITALSFYGMSTFEGPMMSTRAVNSLSHY	248
<i>Rhizobium leguminosarum</i>	: SCAMDKVR	TDPIIRMMIIAIAFYGMSTFEGPMMSVKTVNLSHY	310
<i>Rhodopirellula baltica</i>	: RGAWHKVA	ADPVLKFFVVGVTFFYGMSTFEGPMMSTRTVNLSHY	307
<i>Bradyrhizobium japonicum</i>	: SCAMDKLR	TDPVLRLVVSVAFYGMSTFEGPMLSTKRSINAFTHY	324
<i>Agrobacterium tumefaciens</i>	: SCAMDKIR	TDPIVRMMVMAVAFYGMATFEGPMMSTRTVNLSHY	310
<i>Vibrio parahaemolyticus</i>	: SCAMHKL	RYDPILRFLIVLSLFYGMSTFEGPMMSTRAVNSLSHY	253
<i>Aromatoleum aromaticum</i>	: SCAMHKL	RDPILKFLITALSFYGMSTFEGPMMSTRTVNLSHY	248
<i>Cupriavidus metallidurans</i>	: SCAMHKL	RDPILKFLVVVLSFYGMATFEGPMMSTRTVNLSHY	255
<i>Rhodopirellula baltica</i>	: RGAWHKVA	ADPVLKFFVVGVTFFYGMSTFEGSMMSTRTVNLSHY	307
<i>Polaromonas naphthalenivorans</i>	: SCAMHKL	RDPILRFLIVLSLFYGMSTFEGPMLSTKRSINAFTHY	253
<i>Acidovorax avenae</i> subsp.	: SCAMHKL	RDPILRFLIVLSLFYGMATFEGPMMSTRTVNLSHY	253
<i>Pseudomonas putida</i>	: SCAMHKL	RDPILRFLVVLSAFYGMSTFEGPMMSTRTVNLSHY	252
<i>Shewanella</i> sp.	: SCAMHKL	RDPVLRFLVVLSLFYGMSTFEGPMMSTRTVNLSHY	254
<i>Methylobacterium nodulans</i>	: SCAMDKLR	TDPIRLMMVVSIAFYGMATFEGPMMSTRTVNLSHY	324
<i>Rhodopseudomonas palustris</i>	: SCAMHKL	RDPVLRMLVVSIAFYGMSTFEGPMLSVKAVNALSHY	322
<i>Polynucleobacter necessarius</i> subsp.	: SCAMYKL	RDPILKFLVVVLSFYGMSTFEGPMMSTRTVNLSHY	254
<i>Magnetococcus</i> sp.	: SCAMDKLR	TDPIIRFMVVSLSFYGMSTFEGSMMSTRTVNLSHY	253
<i>Marinobacter aquaeolei</i>	: SCAMHKL	RDPILRFLVVLSLFYGMSTFEGPIMSTKEVNLSHY	252
<i>Methylocella silvestris</i>	: SCAMDKLR	TDPVLRLVVLSIAFYGMSTFEGPMMSTRTVNLSHY	326
<i>Thioalkalivibrio</i> sp.	: SCAMHKL	RDPILRFLIVLSLFYGMSTFEGPMLSTKRTVNLSHY	249
<i>Verminephrobacter eiseniae</i>	: SCAMHKL	RDPVLRFLIVLSLFYGMSTFEGPMMSTRTVNLSHY	260
<i>Delftia acidovorans</i>	: SCAMHKL	RDPILRFLIVLSLFYGMSTFEGPMMSTRTVNLSHY	253
<i>Pseudoalteromonas atlantica</i>	: SCAMHKL	RDPILRFLVVLSLFYGMSTFEGPMMSTRTVNLSHY	243
<i>Brucella melitensis</i>	: SCAMDKVR	TDPIIRLMVAAIAFYGMATFEGPMLSTRAVNSLSHY	308
<i>Parvibaculum lavamentivorans</i>	: SCAMDKLR	TDPVIRMLVVSVAFYGMSTFEGPVMSTRAVNSLSHY	328
<i>Xanthobacter autotrophicus</i>	: SCAMDKLR	TDPIIRMMVAVAFYGMSTFEGPMMSVKAVNSLSHY	330
<i>Alkalilimnicola ehrlichei</i>	: SCAMHKL	RDPILKFLIVLSLFYGMSTFEGPMMSTRTVNLSHY	249
<i>Leptothrix cholodnii</i>	: SCAMHKL	RDPILKFLIVLSLFYGMSTFEGPMMSTRTVNLSHY	254
<i>Ralstonia pickettii</i>	: SCAMHKL	RDPILKFLVVVLSFYGMATFEGSMMSTRTVNLSHY	255
<i>Ochrobactrum anthropi</i>	: SCAMDKVR	TDPIIRLMVAAIAFYGMATFEGPMLSTRAVNSLSHY	308
<i>Halothiobacillus neapolitanus</i>	: SCAMWKLR	SDPIMLFLITAMAFYGMSTFEGPMMSLKSVNALSHY	258
<i>Chthoniobacter flavus</i>	: RGAWHQL	RDPVLKFFAAGVTFFYGMATFEGPLLSTRTVNLSHY	254
<i>Opitutus terrae</i>	: RGAWHKL	RDPVLKFFAAGVTFFYGMATFEGPLLSTRAVNALCHY	252
<i>Diaphorobacter</i> sp.	: SCAMHKL	RDPILRFLIVLSLFYGMSTFEGPMMSTRTVNLSHY	253
<i>Variovorax paradoxus</i>	: SCAMHKL	RDPILRFLIVLSLFYGMSTFEGPMMSTRTVNLSHY	253
<i>Comamonas testosteroni</i>	: SCAMHKL	RDPILRFLIVLSLFYGMSTFEGPMMSTRTVNLSHY	253
<i>Psychromonas ingrahamii</i>	: SCAMHKL	RDPILRFLVVLSLFYGMSTFEGPMMSTRTVNLSHY	261
	*** :	***: : : : : : : : : : : : : : : *	

Figure 1. 10 Amino acid alignment of heme-copper oxidase subunit I (accession numbers in parenthesis). *Rhd. capsulatus* (AAC46108), *Rhd. sphaeroides* (ATCC 17029), *P. denitrificans* (PD1222), *A. sp.* (BH72), *Rh. leguminosarum* (WSM2304), *R. baltica* (strain SH 1), *Br. japonicum* (*Bradyrhizobium* strain 110spc4), *A. tumefaciens* (*Agrobacterium* strain T37), *V. parahaemolyticus* (*Vibrio* strain 16), *A. aromaticum* (EbN1), *C. metallidurans* (CH34), *P. naphthalenivorans* (CJ2), *Ac. avenae* subsp. (*Acidovorax* subsp. *citrulli* AAC00-1), *P. putida* (W619), *Sh. sp.* (MR-4), *M. nodulans* (ORS 2060), *R. palustris* (BisA53), *P. necessarius* subsp. (*Polynucleobacter* subsp. *asymbioticus* QLW-P1DMWA-1), *M. sp.* (MC-1), *M. aquaeolei* (VT8), *M. silvestris* (BL2), *Th. sp.* (HL-EbGR7), *V. eiseniae* (EF01-2), *D. acidovorans* (SPH-1), *Ps. atlantica* (T6c), *Br. melitensis* (biovar Abortus 2308), *P. lavamentivorans* (DS-1), *X. autotrophicus* (Py2), *Al. ehrlichei* (MLHE-1), *L. cholodnii* (SP-6), *R. pickettii* (12J), *Oc. anthropi* (ATCC 49188), *H. neapolitanus* (c2), *Ch. flavus* (Ellin428), *Op. terrae* (PB90-1), *Dph. sp.* (TPSY), *V. paradoxus* (S110), *C. testosteroni* (KF-1), *P. ingrahamii* (37). Transmembrane helices for the *R. capsulatus* *cbb₃* oxidase are indicated by cylinders on the top of alignment. Strictly and almost strictly conserved amino acid residues are shaded in green. Alignments were made by using BioEdit and manually adjusted.

1.2.12. The Aim of the Present Study

1.2.12.1. What do the Decoupled Mutants of Cytochrome Oxidase Tell Us?

Recently three distinct families of HCOs, type A, B and C, were defined based on sequence alignments and structural information. (i) Type A (Mitochondrial-like oxidase, *aa₃*-type oxidase), (ii) type B (*ba₃*-like oxidases) and (iii) type C (*cbb₃*-type oxidases) [4]. Only catalytic subunit is homologous in all members of the superfamily. Subunit I is approximately 500 amino acids long and forms at least 12 transmembrane helices; generally with short inter helical loops. The C-family (*cbb₃* oxidases) is the second most abundant oxygen reductase family after A-family, although subunit I of the *cbb₃*-type oxidase has a lower degree of similarity at the amino acid sequence level being less than 20% [19]. Cytochrome *cbb₃* oxidases are quite distinct from other bacterial HCOs in terms of their strategy for receiving electrons, the heme prosthetic group present in the active site and their affinity for oxygen.

Site-directed mutagenesis experiments [64] and X-ray crystal structures [65] of the A- and B-family revealed the presence of two protein-input channels, named the D- and the K-pathways [66]. These two pathways provide 4 protons/ O_2 that are consumed at the active site to generate 2 H_2O , and another 4 protons/ O_2 that are pumped across the membrane [54]. Sequence alignments show that all known type A and B oxidases contain conserved amino acids in D- and the K-pathways. In the *P. denitrificans* structure model, whereas K pathway includes lysine (K) 362, D pathway includes aspartate (D) 132 and glutamate (E) 286

amino acids [67]. Mutations in which either of these acidic residues is related by their corresponding amides resulted in severe inhibition of enzyme activity. Glutamate residue in *aa₃* type oxidase of *P. denitrificans* is one of the best conserved residues among the HCOs, and mutations at this locus have been shown to block the oxygen reaction and lowered the enzyme activity [57]. However, nothing is known about proton channels in the *cbb₃* oxidases. In contrast, sequence alignments [3] show these residues to be missing in all of the type C (*cbb₃*) oxidases, raising the important question of whether the *cbb₃*-type oxidases might employ a different catalytic mechanism for facilitating the proton pump. The cytochrome *cbb₃* oxidases have a glycine or alanine at the equivalent position in sequence alignments. This situation presents a possibility to identify shared structural features that are perhaps from a different part of the overall structure but serve the same function of coupling proton transport and electron transfer.

When amino acid sequence of catalytic subunit of *cbb₃* type oxidase from 40 organisms was aligned, it was observed that D361, E380 and K387 (*R. capsulatus* numbering) residues were conserved in the helix IX (Fig.1.10). The functionally critical histidine-tyrosine cross-link of active site has been predicted and verified to exist in the *aa₃*- and *ba₃*-type oxidases. This histidine-tyrosine structure is also present in *cbb₃*-type oxidases although tyrosine derives from the different transmembrane helix [6, 10, 68]. Similarly thermodynamic and spectroscopic properties of the redox centers in cytochrome *c* oxidase indicated that histidine-glutamate/aspartate structural motifs found in a variety of enzymes. A sequence comparison of the catalytic subunits shows that this glutamate residue

is not conserved within VIII. helix of *cbb₃*-type oxygen reductase. However, homology modeling has suggested that E380 located within helix IX of subunit I and fully conserved among the *cbb₃*-type oxygen reductases, might fulfill this function. Therefore, homology modeling has suggested that E380 located in the helix IX and fully conserved among the *cbb₃*-type oxidases, might replace the missing glutamate in the active site. It was thought that these three conserved residues, D361, E380 and K387 in helix IX of *cbb₃*-type oxidase in *R. capsulatus* might take the role of the D- and K- channels. There is no crystal structure available for any type C oxidase. However, without a structure or any mutagenesis data is difficult to conclude whether these three conserved residues in *cbb₃*-type oxidases might take the role of the D- and K- channels, or have developed a completely different method for proton translocation.

Our goal is to understand molecular mechanism of proton pumping channel and to address the question whether the C type (*cbb₃*) oxidases might employ a different catalytic mechanism in *Rhodobacter capsulatus*. In this work, three conserved residues, D361, E380 and K387, within helix IX of subunit I of the *cbb₃*-type oxygen reductase of *R. capsulatus* were substituted for leucine (L), glutamine (Q) and methionine (M) respectively by site directed mutagenesis. The effects of these mutations on enzyme activity were evaluated by NADI staining.

1.2.12.2. The Possible Expectations

There is no three-dimensional structure available for any *cbb*₃-type oxidases. Reasonable conclusions from this work that also are consistent with the current literature are (i) all heme-copper oxygen reductases possess a common active-site structure that catalyzes similar O₂-reduction chemistry, (ii) the proton-loading site and unidirectional gate(s) are likely to derive from the common active site structure, (iii) future efforts are likely to reveal additional structural diversity in the channels by which protons move from the “inside” to the active site, and (iv) the proton channels are likely to be conveyors of protons rather than being specifically involved as gating elements, (v) there is no detailed studies of proton conduction mechanism of the D-channel of *cbb*₃-type cytochrome oxides. The detailed chemistry of these processes remains mysterious [69].

CHAPTER 2

MATERIALS AND METHOD

2.1. The Bacterial Growth Conditions and Plasmid DNA Isolation

The bacterial strains and plasmids used in this work are listed in Table 2.1. *Escherichia coli* strains were grown in Luria-Broth (LB) medium supplemented with appropriate antibiotics (ampicillin, 100µg/ml; kanamycin, 50µg/ml; tetracycline, 12.5 µg/ml per ml, respectively) for plasmid selection and maintenance as described previously [70, 71] (Appendix- A).

R. capsulatus strains were grown on either Sistrom's minimal medium A (Med A) [72] or MPYE enriched growth medium [70] supplemented with appropriate antibiotics (Tetracycline, 2.5µg/ml; kanamycin, 10 µg/ml; or ampicillin, 10 µg/ml as needed) [70] at 35 °C chemoheterotrophically under aerobic conditions in the dark on either the petri plates or liquid cultures shaken at 100 rpm. A single colony was used to inoculate for 5-10 ml culture from LB medium with suitable antibiotic provided to each sample and grown at 37 °C, with agitation at 175 rpm for 12-16 hours. The 5-10 ml cultures were prepared depending on the strains which containing high copy or low-copy number of plasmids. DNAs were isolated using Wizard SV Minipreps DNA Purification Kit (Promega cat. #: A1330), GeneJET™ Plasmid Miniprep Kit (Fermentas cat. #: K0502) and the protocol of manufacturer were followed.

Table 2. 1 Bacterial strains and plasmids

<u>Strains</u>	<u>Genotype</u>	<u>Phenotype</u>	<u>Reference</u>
<i>E. coli</i>			
XL1-Blue	rec A end A 1 gyr A986 thi-1hsdr17supE44 rel A1 lac [F' proAB lacIq ΔM15 Tn 10(Tetr)]		Stratagene
XL1-Blue/ pMOZI	2.8 kbp <i>XhoI ccoNO</i> ' fragment in pBluescript SK+ DNA	Amp ^r	[74]
XL1-Blue/pMOZID361N		Amp ^r	This work
XL1-Blue/pMOZIE380Q		Amp ^r	This work
XL1-Blue/pMOZIK387M		Amp ^r	This work
HB101	F-proA2 hsdS20 (Rb-, mB-) recA13 ara14 lac Y1		[73]
HB101/pACYC177	xy15 galK2 rpsL20 supE44 rpsL20 supE44 proA2 mtl-1	Amp ^r , Kan ^r	Biolabs
HB101/pMOZII	2.8 kbp <i>XhoI ccoNO</i> ' fragment in pACYC177 DNA	Amp ^r	[74]
HB101/pMOZIID361N		Amp ^r	This work
HB101/pMOZIIIE380Q		Amp ^r	This work
HB101/pMOZIIK387M		Amp ^r	This work
HB101/pMOZIII	0.6 kbp Ppu21I fragment of pMOZII absent	Amp ^r	This work
DH5α	F-, φ80dlacZΔM15, Δ(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rk-, mk+), phoA, supE44, λ-, thi-1, gyrA96, relA1		[75]
DH5α/pOX15D361N		Tet ^r	This work
DH5α/pOX15E380Q		Tet ^r	This work
DH5α/pOX15K387M		Tet ^r	This work
<i>R. capsulatus</i>			
MT1131	<i>crtD121 Rif^r</i>	Wild-type	[37]
MT1131 /pOX15		Tet ^r	[70]
GK32	(Δ <i>ccoNO</i> ::kan)	Kan ^r	[37]

GK32/pOX15D361N	GK32, complemented with pOX15 D361N	Tet ^r	This work
GK32/pOX15E380Q	GK32, complemented with pOX15 E380Q	Tet ^r	This work
GK32/pOX15K387M	GK32, complemented with pOX15 K387M	Tet ^r	This work
<u>Plasmids</u>	<u>Genotype</u>	<u>Phenotype</u>	<u>Reference</u>
pBluescript	SKII+	Amp ^r	Stratagene
pMOZI	pBlue Script SK+ with a 2.8-kbp <i>XhoI</i> insert	Amp ^r	[74]
pMOZID361N	Substitution of aspartate 361 by asparagine	Amp ^r	This work
pMOZIE380Q	Substitution of glutamate 380 by glutamine	Amp ^r	This work
pMOZIK387M	Substitution of lysine 387 by methionine	Amp ^r	This work
pACYC177		Amp ^r , Kan ^r	New England Biolabs
pMOZII	pACYC177 with a 2.6-kbp <i>XhoI</i> insert	Amp ^r	This work
pMOZIID361N	pMOZII replaced 0.6-kbp <i>BsaAI</i> fragment of pMOZID360N	Amp ^r	This work
pMOZIIIE380Q	pMOZII replaced 0.6-kbp <i>BsaAI</i> fragment of pMOZIE380Q	Amp ^r	This work
pMOZIIK387M	pMOZII replaced 0.6-kbp <i>BsaAI</i> fragment of pMOZIK387M	Amp ^r	This work
pOX15	pRK404 with 4.7-kbp <i>BamHI-EcoRI</i> fragment	Tet ^r	[37]
pOX15D361N	pOX15 replaced 2.8-kbp <i>XhoI</i> fragment of pMOZII D361N	Tet ^r	This work
pOX15E380Q	pOX15 replaced 2.8-kbp <i>XhoI</i> fragment of pMOZIIIE380Q	Tet ^r	This work
pOX15K387M	pOX15 replaced 2.8-kbp <i>XhoI</i> fragment of pMOZII K387M	Tet ^r	This work
pRK2013		Kan ^r , helper	[76]

2.2. The Enzyme Digestion of Plasmid DNAs

To clone target insert DNA into plasmid DNA, DNAs were digested with the appropriate endonucleases (Fermentas) by following to the Manufacturers' instruction. For cloning, 2 µg plasmid DNA was digested with 1 µl of restriction enzymes (10 U/µl) in 50-60 µl final volume at 37 °C for 2-3 hours. For fast digestion, 2 µg 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. The name and catalog number of restriction endonucleases are listed at Appendix-E. The pOX15 was digested with fast digestion restriction enzyme at 37 °C for 15 minutes and dephosphorylated at 37 °C for 15 minutes because pRK derivative vectors are sensitive to heating. For the insert control 200 ng of plasmid DNA was digested with 0.5 µl of restriction enzymes (5 U/µl) in 20 µl of final volume at 37 °C for 1 h.

2.3. The Isolation of DNA Fragments from Agarose Gel

To isolate DNA fragments from the 1% agarose gel; the Qiagen QIAquick[®] Gel Extraction Kits (cat. #: 28704), Roche High Pure PCR Product Purification Kit (cat.No. 11 732 668 001) and Fermentas DNA Extraction Kit (cat. # K0513) were used and the protocols of the Manufacturer were followed. Digested DNA solution was loaded on 1% agarose gel and the gel was run to separate DNA fragments. Agarose gel bands containing the desired, DNA

fragments were excised from the agarose gel (minimization of UV exposure was taken care to protect DNAs).

2.4. The Ligation of Insert and Vector DNAs

Fermentas T4 ligase (cat. # EL0335) and Stratagene T4 DNA Ligase (cat. 600011) were used for the ligation reaction. Concentration of vector and insert are determined by either spectrophotometric measurement (OD_{260}) or comparing visually for its value with 1 kbp marker on agarose gel. Amount of the vector and insert as a pmol quantity were calculated by conversion to pmol calculated as $1.52 \text{ pmol} \times \mu\text{g of DNA/kbp of DNA}$. The ligation reaction mixture should contain an equal or higher (generally 4-fold) concentration of foreign DNA than that of vector DNA. X pmol vector, 1.5 μl 10X ligation buffer, 3X pmol insert and 2U-4U T4 Ligase are combined and final volume were completed to 15 μl with ddH₂O. The ligation mixture was incubated at 22 °C for 2 hours when working with pBluescript and pACYC177 derivative vectors, but ligation mixture was incubated at 22 °C for 60 minutes and then at +8 °C for 16 hours. T4 DNA ligase was inactivated by heating reaction mixture at 65 °C for 10 minutes.

2.5. Preparation of the Competent Cells and Transformation

To prepare *E. coli* HB101 and XL1-Blue competent cells, 50 ml LB in a 500 ml flask were inoculated with 500 μl culture of *E. coli* HB101 and XL1-Blue strains grown for overnight respectively. The inoculated cultures were incubated at 37 °C with shaking until $OD_{600} = 0.4-0.5$ (it takes nearly 2 h) and then cell cultures were taken on ice. After this step, the cells must be on ice until heat

shock step. The cells were centrifuged for 10 minutes at 5000 rpm at 4 °C. The supernatant was discarded and pellet was resuspended in 25 ml (1/2 of the culture volume) of ice-cold 100 mM CaCl₂ and then incubated on ice for 30 minutes. Cells were centrifuged for 10 minutes at 5000 rpm at 4 °C. Supernatant was discarded and pellet was resuspended in 2.5 ml (1/20 of the culture volume) of ice cold 100 mM CaCl₂. Competent cells were aliquoted 100 µl into Eppendorf tubes. For stock competent cells, glycerol was added (10% of final conc.). DNA was added to 100 µl competent cells then incubated on ice for 45 min. Heat shock was applied to the sample for 5 minutes at 37 °C. 1 ml LB was added and cells were incubated for 1 h at 37 °C with shaking. Finally, cells were centrifuged for 1 minute at 5000 rpm at RT and resuspend in 100 µl LB and then cells were spread plate contains selective medium and incubated overnight [77].

To prepare *E. coli* DH5α competent cell, 250 ml flask containing 50 ml of SOB broth was inoculated with 500 µl overnight culture of *E. coli* DH5α grown overnight and placed to incubator by shaking at 100 rpm and 37 °C. After nearly 7-8 hours later, the optical density of culture was measured by spectrophotometer. When density of culture had grown to 0.6 at OD₆₀₀, cells were taken on ice from the shaker. The competent cell containing cultures were transferred to 50 ml cold Falcon tubes and waited for 10 minutes on ice bath. Centrifugation step was followed at 2500 ×g and 4°C for 10 minutes. After supernatant was discarded, pellets were dissolved 6.4 ml cold TB buffer and waited for 10 minutes on ice. Centrifugation step was repeated at 2500 ×g and 4°C for 10 minutes. The supernatant was discarded again and pellet was dissolved in 1.6 ml cold TB buffer and added DMSO by gently swirling to 7% (112 µl). Heat shock was

applied for 10 minutes on ice. And cells were transferred to sterile and cold Eppendorf tubes each containing a volume of 200 µl.

2.6. The Preparation of Mutant Plasmid DNAs

2.6.1. PCR-Based Site-Directed Mutagenesis

The Quick Change Site-Directed Mutagenesis (Stratagene) protocol was used to make target mutations. This method was performed using *Pfu* DNA polymerase (MBE Fermentas) and a thermal temperature cyclers (Techne TC-3000- Appendix F). *Pfu* DNA polymerase replicates both plasmid strands with high fidelity and without displacing the mutant oligonucleotide primers. The basic procedure utilizes a supercoiled double-stranded DNA vector with an insert and two synthetic oligonucleotide primers containing the desired mutation. The oligonucleotide primers, each of which complementary to opposite strands of the vector are extended during temperature cycling by using *Pfu* DNA polymerase.

Quick Change™ Site Directed Mutagenesis protocol was used to substitute three target amino acids (Table 2.2), aspartate (D361), glutamate (E380) and lysine (K387) for asparagines (N361), glutamine (Q380) and methionine (M387) by using forward and reverse primers for each mutations (Table 2.3) and pMOZI as a template DNA [44]. The sample and control reactions were prepared (Table 2.4) for PCR by using forward and reverse mutagenic primers. All reaction mixtures were incubated at the conditions defined in Table 2.5 for D361N, E380Q and K387M reactions and the control reaction. Following 16 temperature cycling, 10 µl of PCR products from sample and control reaction were loaded on

1% agarose gel and electrophoresed for 1 h to visualize the PCR product. After visualization of PCR products, the products were treated with *DpnI*, to digest the parental template DNA and to select the PCR synthesized DNA. Following the PCR product digestion, The *DpnI* treated PCR product was transformed into XL-1 blue cells. Finally, to select transformants that had desired mutations, DNA was isolated from four transformants for each mutation to be sequenced. The strategy used to select transformants was summarized in Figure 2.1.

Table 2. 2 The nucleotide and amino acid sequence of the subunit I of the *cbb₃* type oxidase in *R. capsulatus*^a.

```
>RRC04162 1D09-1F02_110436_112031
ATG TGG GAT TAC GTC AAG CTT GTC GCG CTT GGC GTC GTG GTG GCT ATT GCC GCT TAC GCC
M W D Y V K L V A L G V V V A I A A Y A
GCC AGT CAG GCG CGA GAT TTG CCG TAT ATG GTG AAC ATG GTC GAG GTC GCG CTG GCG GCC
A T Q A S D L P Y M V N M V E V A L A A
GTG ATC GCG TTC ATC TGG GTG CTG CGC ACC ATG GGG GAC GCC AAA CCC TCG AAA GAC GAG
V I A F I W V L R T M G D A K P S K D E
TAT TTC GAC GGC GTG ATC CGC GCC GGC GTG ATC GCA ACC ACC TTC TGG GGG ATC GTC GGC
Y F D G V I R A G V I A N N F W G I V G
TTT CTC GTG GCC GTC ATC ATC GCC TTC CAG CTT GCC TTC CCC GCG CTG AAC CTT GAA TTC
F L V A V I I A F Q L A F P A L N L E F
GGC AAC GGG ATG CTG AAC TTC GGG CGC CTG CGT CCG CTG CAC ACC TCG GCG GTG ATC TTC
G N G M L N F G R L R P L H N S A V I F
GCC TTT GGC GGC AAC GCC CTG ATC GCC TCG GCC TTC TAT GTC GTG CAG CGC ACC TCG GCG
A F G G N A L I A S A F Y V V Q R N S A
GCG CGT CTC TTC GGG GGC ACG GCG CTT GGC TGG TTC GTG TTC TGG GGC TGG CAG CTG ATC
A R L F G G T A L G W F V F W G W Q L I
ATC GTC ACC GCC GCG ACC TCC TAT CTG CTG GGC GGC TCG CAG GGC AAG GAA TAT GCC GAG
I V T A A T S Y L L G G S Q G K E Y A E
CTG AAC TGG CAT CTC GAC ATC CTC GTC GCG ATC GTC TGG GTG GCC TAT CTG ATC GCC TTC
L N W H L D I L V A I V W V A Y L I A F
CTC GGC ACG ATC TTC AAG CGC AAG GAA CCC CAC ATC TAC GTG GCG AAC TGG TTC TAC CTG
L G T I F K R K E P H I Y V A N W F Y L
TCC TTC ATC GTG ACG ATC GCC ATG TTG CAC ATC GTC AAC AAC CTG GCC GTT CCG GTC TCG
S F I V T I A M L H I V N N L A V P V S
ATC TTC GGC ACC AAA TCG GTG CAG CTG ATG GCG GGC GTG CAG GAT GCG ATG ACG CAA TGG
I F G N K S V Q L M A G V Q D A M T L W
TGG TAC GGC CAC AAC GCC GTG GGC TTC TTC CTC ACC GCG GGC TTT CTG GGC ATG ATG TAT
W Y G H N A V G F F L T A G F L G M M Y
```

TAC TTC GTG CCG AAA CAG GCC GAG CGT CCC GTC TAT TCC TAC AAG CTC TCG ATC GTC CAC
Y F V P K Q A E R P V Y S Y K L S I V H
TTC TGG GCG CTG ATC TTC CTC TAC ATC TGG GCC GGT CCG CAC CAC CTG CAT TAT ACC GCG
F W A L I F L Y I W A G P H H L H Y T A
CTG CCC GAC TGG GCC TCG ACG CTG GGC ATG GTG ATG TCG GTG ATC CTG TGG ATG CCG TCC
L P D W A S T L G M V M S V I L W M P S
TGG GGC GGC ATG ATC AAC GGC CTG ATG ACG CTT TCG GGC GCC TGG GAC AAG CTG CGC ACC
W G G M I N G L M T L S G A W D K L R T
GAT CCG GTG ATC CGG ATG ATG GTG GTT TCG ATC GGC TTT TAC GGC ATG TCG ACC TTT **GAA**
D P V I R M M V V S I G F Y G M S T F E
GGC CCG ATG ATG TCG ATC **AAG** GCG GTC AAC TCG CTC TCG CAC TAC ACG GAC TGG ACC ATC
G P M M S I **K** A V N S L S H Y T D W T I
GGT CAC GTG CAT TCG GGC GCG CTG GGC TGG AAC GGC ATG ATC ACC TTC GGC ATG CTC TAC
G H V H S G A L G W N G M I T F G M L Y
TTC CTG ACG CCG CGC CTG TGG GGC CGT TCG GGG CTT TAC TCG CTG AAA CTG GTC AGC TGG
F L T P R L W G R S G L Y S R K L V S W
CAC TTC TGG CTC GCC ACC ATC GGG ATC GTG CTT TAC GCC **TC** TCG ATG TGG GTG TCG GGC
H F W L A T I G I V L Y A S S M W V S G
ATC ATG GAA GGC CTG ATG TGG CGT GAA GTC GAT GCG CAG GGC TTC CTG GTG AAC GGT TTC
I M E G L M W R R V D R Q G F L V N G F
GCC GAC ACC GTG GGC GCC AAG TTC CCG ATG AAC GTC GTG CGT GGC GTC GGG GGT GTG CTG
A D T V G A K F P M K V V R G V G G V R
TAT CTG ACC GGC GGT CTG ATC ATG GCC TAC AAC CTT TGG GCC ACG GTC GCG AAA CAA CCG
Y V T G G R I M A Y K L W A T V A K E P
AAG ACG GCC AAT CTC GCC GTT GCG GTT CCG GCG GAA
K T A I L A V A V P A E

^a The target conserved amino acids codons, D361, E380 and K387, respectively were highlighted.

Table 2. 3 The forward and reverse primers used for mutating of the desired amino acids.

Primers	The sequence of the primers
D361NF	5'GGA CAA GCT GCG CAC CCT GCC GGT GAT CCG G-3'
D361NR	5'CCG GAT CAC CGG CAG GGT GCG CAG CTT GTC C-3'
E380QF	5'GGC ATG TCG ACC TTT CAG GGC CCG ATG ATG TCG-3'
E380QR	5'CGA CAT CAT CGG GCC CTG AAA GGT CGA CAT GCC-3'
K387MF	5'-CCG ATG ATG TCG ATC ATG GCG GTC AAC TCG C-3'
K387MR	5'-GCG AGT TGA CCG CCA TGA TCG ACA TCA TCG G-3'

Table 2. 4 The reaction conditions of site-directed *in vitro* mutagenesis

The reactants	The sample reaction	The control reaction
Template DNA	2 ul	2 ul
Buffer	5 ul	5 ul
Primer	1 ul	1 ul
Primer	1 ul	1 ul
ddH ₂ O	39.6 ul	40.6 ul
dNTP mix	0.4 ul	0.4 ul
Enzyme	1 ul	-
Total reaction volume	50 ul	50 ul

Table 2. 5 The cycling parameters for the Quick Change site-directed *in vitro* mutagenesis method.

The reactants	The temperature and cycle numbers	The time period
Initial denaturation	95°C	30 sec
Denaturation	95°C	30 sec
Annealing	68°C	60 sec
Extension	72°C	15 min
Go to cycle	2	
Cycle number	16	
Hold at	4°C	

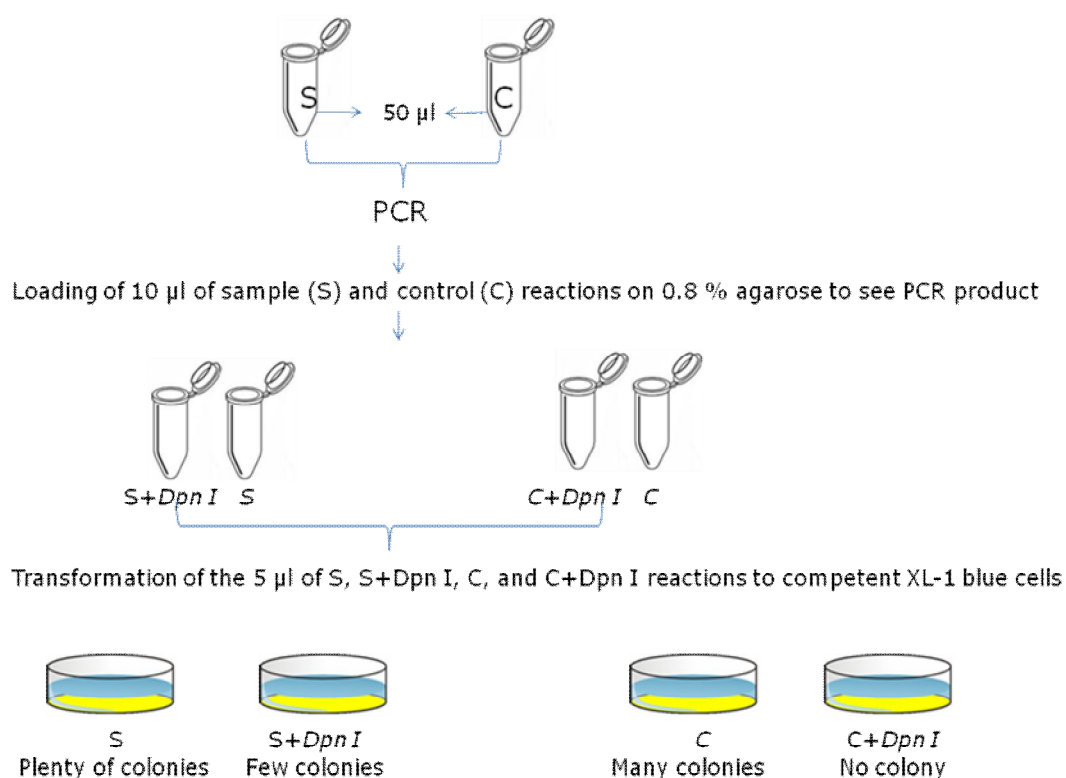


Figure 2. 1 Strategy for selecting mutagenized transformants [38].

2.6.2. The Confirmation of Desired Mutation

After site-directed mutagenesis works, plasmid DNAs, carrying desired mutations, (pMOZID361N, pMOZIE380Q and pMOZIK387M) were partially sequenced (0.6 kbp part of the 2.8 kbp *NO'* insert DNA fragment of pOX15) using the primer 578-F (5'- GGT GGGC CTA TCT GAT CG- 3') by MWG-BIOTECH and Iontek (Istanbul) to be sure desired mutations are present the clones.

2.6.3. The Construction of pMOZII Mutants

The pMOZIII was chosen as a vector DNA because of it was ready to use. This plasmid was obtained by separation of 0.6 kbp healthy fragment of pMOZII [44] to reorientation into it, mutation containing part of pMOZI*. To prepare vector and inset DNA, pMOZIII (6.8 kbp) and pMOZI* DNAs were digested with *Ppu2II* (*BsaAI*) (Fermentas). *Ppu2II* (*BsaAI*) digested sample DNAs were loaded on 1% agarose gel, electrophoresed and then 0.6 kbp fragment of pMOZI* and 6.2 kbp fragment of pMOZIII were isolated from the agarose gel. Linear fragment of pMOZII was dephosphorylated with shrimp alkaline protease (SAP, Fermentas) to prevent self ligation of linear vector during the ligation reaction (Fig. 2.2) for 1 hour at 37 °C.

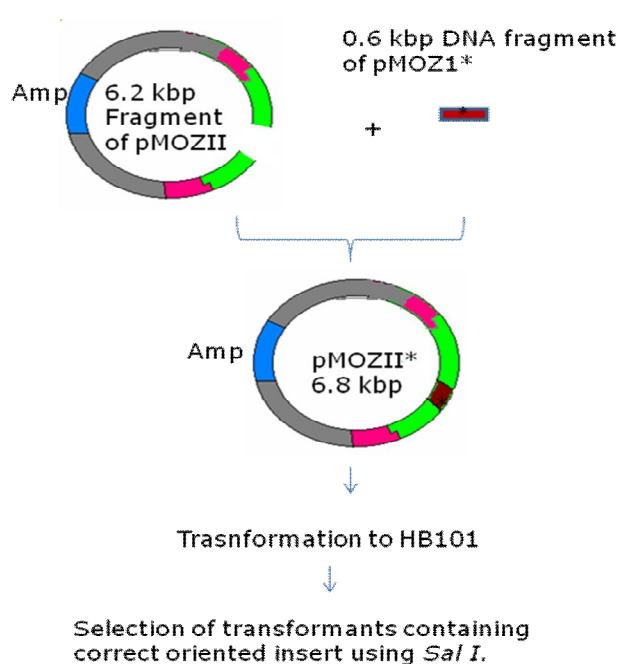


Figure 2. 2 The replacement of the 0.6 kbp mutated fragment from pMOZI* with 0.6 kbp wild one of pMOZII.

During ligation reaction, 0.6 kbp DNA fragment could be inserted into 6.2 kbp dephosphorylated vector in two orientations. The correct oriented insert was

detected by the digestion of transformant plasmid DNA with *Sa*II at 37 °C for 2 hours. *Sa*II has two restriction sites in plasmid DNA; the first site is in the vector DNA and second one is DNA fragment. If insert was oriented in right orientation, 1.5 kbp and 5.3 kbp fragments would be obtained (Figure 2.3). If *Sa*II digestion, insert was wrong orientated, 1.1 kbp and 5.7 kbp fragments would be obtained. The clones containing desired mutation were called HB101/ pMOZIID361N, HB101/ pMOZIIIE380Q and HB101/ pMOZIIK387M and stocked at -80 °C

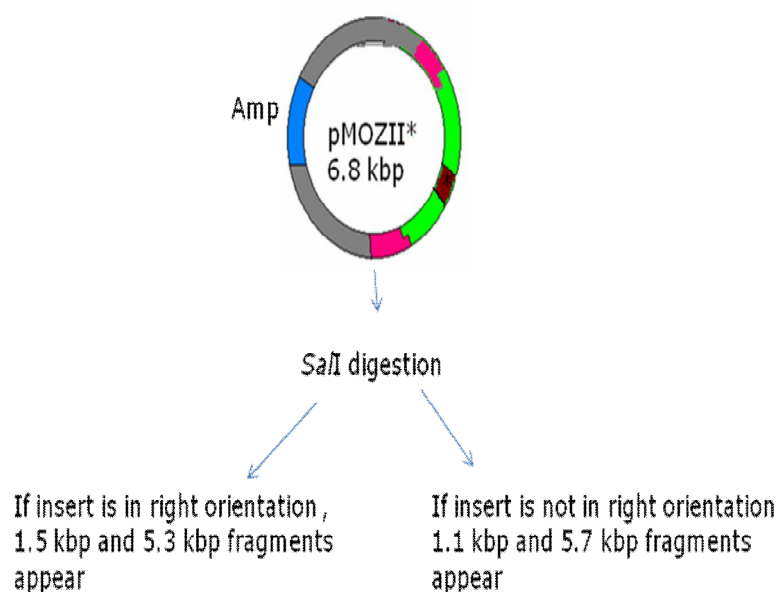


Figure 2. 3 The control of the 0.6 kbp insert orientation. The pMOZII* DNA was digested with *Sa*II to select transformant having correct oriented insert.

To replace 2.8 kbp DNA fragment from pMOZII* with that of pOX15, pMOZII*s plasmid DNAs were digested with *Xho*I restriction enzyme. The resulting fragments, 2.8 kbp and 4.0 kbp, were separated with 1% agarose gel by electrophoresis and then 2.8 kbp was isolated from the gel by using the QIAquick gel extraction kit. On the other hand, pOX12 DNA was digested with *Xho*I (Fermentas-fast digestion) for 30 minutes and then incubated with shrimp alkaline protease (SAP-Fermentas) for 45 minutes to prevent from self ligation. The pOX12 is a vector 2.8 kbp fragment of which was taken out from pOX15

plasmid. Digested pOX12 was loaded on the 1% agarose gel and then 12.5 kbp dephosphorylated fragments were isolated from the gel. The amount of vector and insert DNA was determined by comparison of the fragment with marker bands' amounts of which were known. Gel purified 2.8 kbp fragment was mixed with 12.5 kbp dephosphorylated vector in 20 μ l ligation reaction. The mixture was incubated at 22 °C for 30 minutes and then kept on ice (Figure 2.4). And half of the ligation mixes were transformed into 100 μ l HB101 cells to increase transformation efficiency. The transformants were selected based on the clone size, 15.3 kbp.

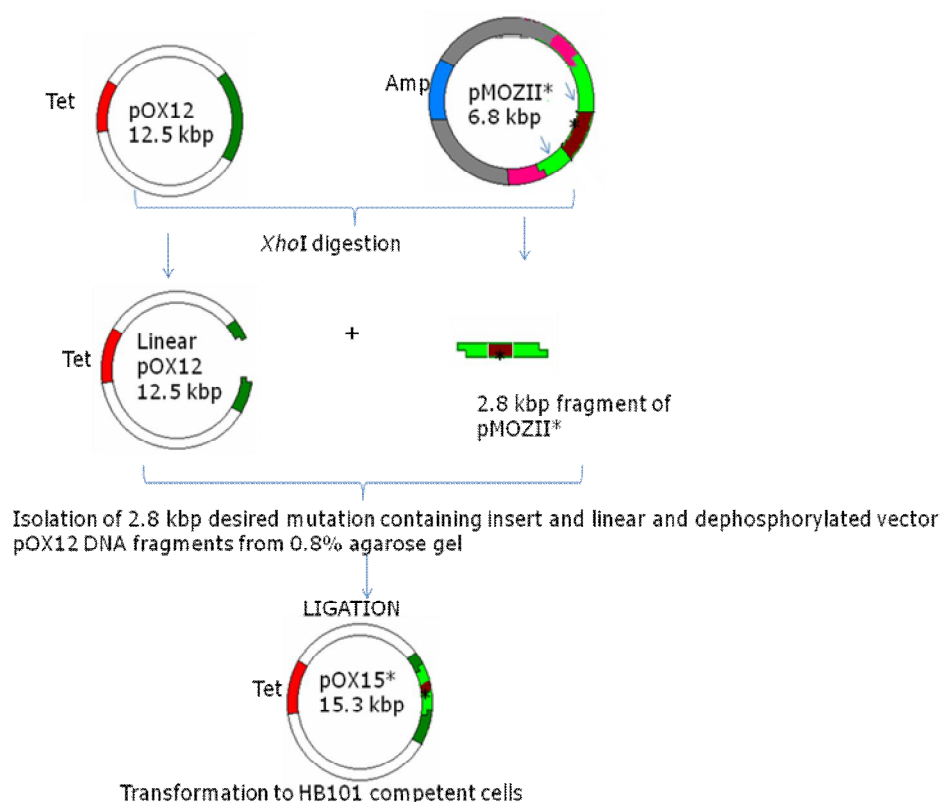


Figure 2. 4 The replacement of the 2.8 kbp mutated fragment of pMOZII* with that of the pOX15.

To select correct oriented insert, pOX15*clones were digested with *Hind*III restriction enzyme at 37°C for 1 hour. *Hind*III enzyme, has one restriction site on vector and one restriction site on insert. After digestion, depending on

right or wrong insert orientation, 3.6 kbp and 11.7 kbp fragments or 2.8 kbp and 12.5 kbp fragments can be obtained (Figure 2.5). The new clones each of which contains one of the three desired mutations were kept and named as HB101/pOX15D361N, HB101/pOX15E380Q and HB101/pOX15K387M at -80 °C.

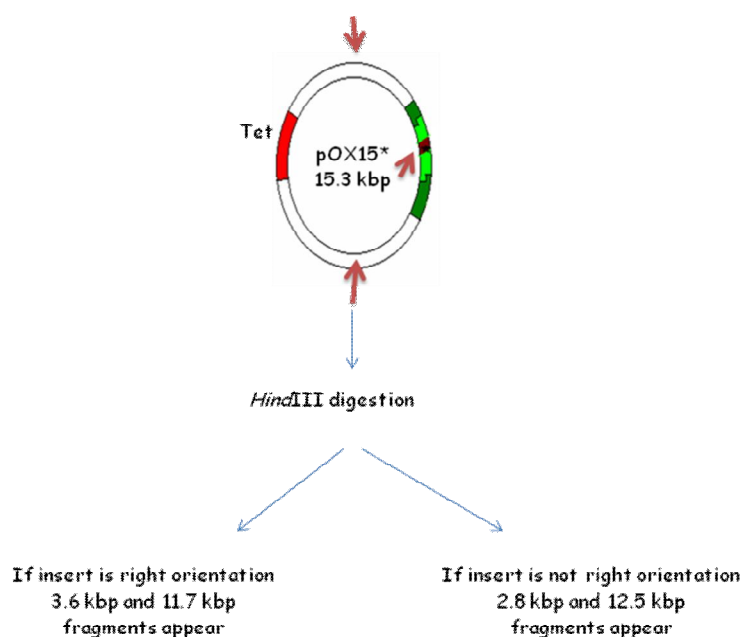


Figure 2. 5 The detection right oriented clone of pOX15* with *HindIII*.

2.6.4. Conjugation (Triparental Mating)

Conjugation is the most efficient and generally applicable system for transfer of plasmid DNA into *R. capsulatus*. GK32 strain of *R. capsulatus*, lacking *ccoNOQP* gene (*ccoNO*::kan), complemented with three recombinant plasmids (Fig. 2.6) that contain desired mutations by Triparental Mating procedure described by [64]. Two days before the mating, 1 ml Med A medium, containing kanamycin was inoculated with a single colony of the recipient *R. capsulatus* GK32 strain. The day before the mating 1 ml culture was added to 10 ml Med A medium containing kanamycin and grown at 35 °C with 90 rpm for 18-

24 h by shaking. The day before the mating, 5 ml LB medium containing kanamycin was inoculated with a single colony of the helper strain pRK2013/HB101 from a fresh plate. 10 ml LB medium containing tetracycline was inoculated with the donor strains that carry the desired mutations (pOX15/D361N, pOX15/E380Q and pOX15/K387M) from a single colony on a fresh plate and grown 12-16 h at 37 °C with shaking (175 rpm). Next morning, the mature cells were centrifuged at 37 °C at 4000 rpm for 10 minutes. The supernatant was discarded and pellet was resuspended in 1 ml Med A. During this period, the required numbers of MedA plates (number of matings plus a control) were dried at an incubator for 30 min. 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 $OD_{600} = 1$

Measuring the OD of cultures at 1/10 dilution is the best to determine the cell density. After determination of cell density, the cells were washed twice with 1 ml Med A to remove all antibiotics at 25°C by 10.000 rpm for 3 min. The cells were resuspended in a volume of Med A density of which was 10^{10} /ml. Then, 100 µl of recipient *R. capsulatus* and 20 µl of each of helper and donor *E. coli* were added and mixed thoroughly. Plates were incubated at 35°C for 24 hours. Approximately 24 hours later, 1ml MedA was pipetted on each spot and the cells were scraped off the agar surface into the MedA broth. Then, the cells were collected at the one edge of the plate and transferred into Eppendorf tubes.

After the cells were centrifuged at 10.000 rpm and at 25°C for 1 min, the supernatant was discarded, and the cells were washed in 1 ml Med A. The washed cells were resuspended in 400 µl Med A. 2 µl, 3 µl and 50 µl of the cell suspension were spread on Med A plates with appropriate antibiotics and incubated for 2-3 nights. At least 4 transconjugants from each cross were purified by picking from the center of well separated colonies and streaked on Med A medium containing plate.

GK32, lacking functional *ccoNOQP* structural gene of the *cbb₃* oxidase enzyme, was complemented with pOX15* plasmid (Fig. 2.7) by three parental conjugation in the presence of a helper strain; pRK2013. Transconjugants were selected on the basis of tetracycline resistance on tetracycline containing medium. The selected transconjugants were stored at -80°C.

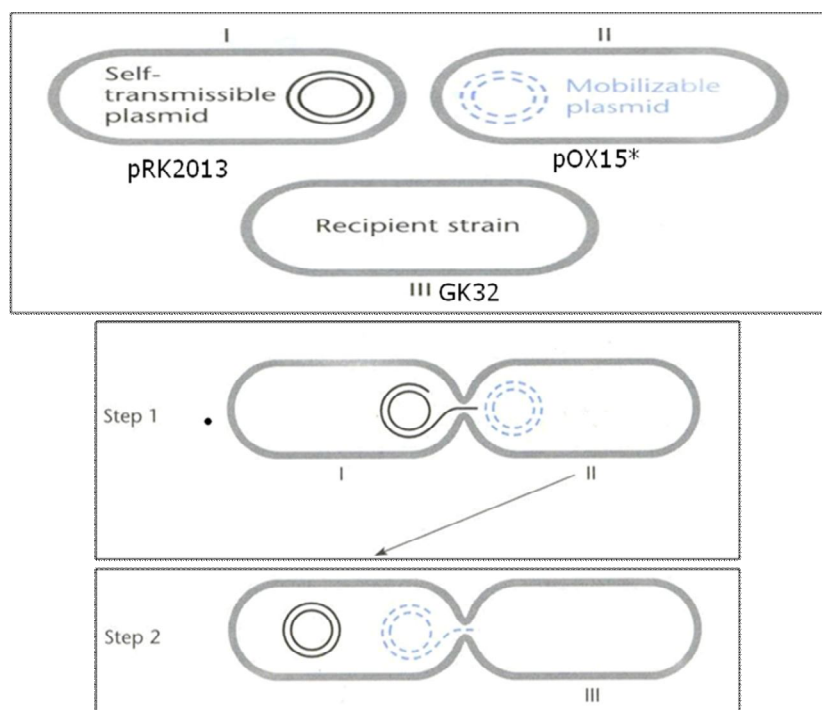


Figure 2. 6 The triparental mating among the pRK2013/HB101, DH5α/pOX15*D361, DH5α/pOX15*E380 and DH5α/pOX15*K387 and GK32.

Donor cell : *E. coli* pOX15*/HB101, Tet^R
 Recipient cell : *R. capsulatus* strain, GK32, [$\Delta(\text{ccoNO}':\text{kan})$]
 Helper cell : *E. coli* pRK2013/HB101, Kan^R

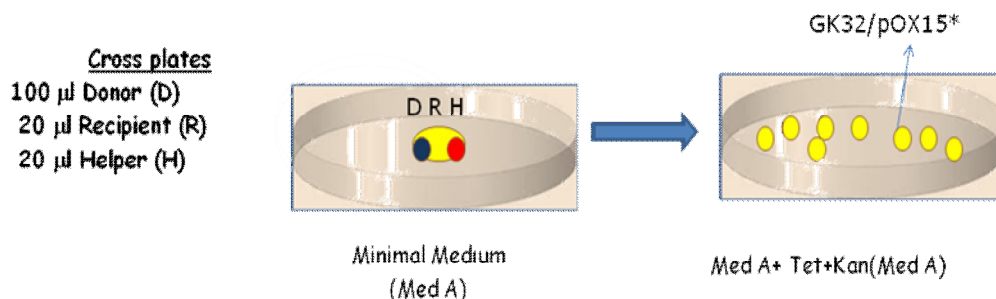


Figure 2. 7 The complementation of the GK32 strains of *R. capsulatus* with pOX15*mutants; pOX15D361, pOX15E380 and pOX15K387.

2.6.5. NADI Staining -Analysis of the Cytochrome *cbb*₃ Oxidase Activity

The presence of only one-type of cyt *c* oxidase in *R. capsulatus* allows efficient utilization of the NADI (α -Naphthol + DMPD + O₂ $\rightarrow$ Indophenol Blue + H₂O) reaction [78] for identification of mutation affecting cyt *cbb*₃ oxidase activity. Transconjugants, GK32/D361N, GK32/E380Q and GK32/K387M, were tested for their cyt *c* oxidase activity by being overlaid with a 1:1 (v/v) mixture of 35 mM α -naphthol in ethanol and 30 mM *N,N*-dimethyl-*p*-phenylenediamine in H₂O. Colonies grown on Med A medium turn blue within 30 seconds if they contain an active cyt *cbb*₃ oxidase.

CHAPTER 3

RESULTS

3.1. The Substitution of Target Amino Acids into the Desired Forms

The pMOZI (Figure 3.1) DNA [38] was used as template DNA. Three target amino acids, aspartate (D361), glutamate (E380) and lysine (K387), were substituted for asparagine (N361), glutamine (Q380) and methionine (M387), respectively by Quick ChangeTM site-directed mutagenesis. To prepare two sets of PCR products, each PCR products from sample and control reactions were divided in two tubes equally (20 µl). *DpnI* enzyme was only added to first set of the tubes to digest the parental template DNA. To visualize PCR product 10 µl from samples, D361, E380 and K387, and control reaction were loaded on 1% agarose gel and electrophoresed for 1 hour (Figure 3.2). *DpnI* was added to the PCR product to digest the parental template DNA. All PCR products from D361, E380 and K387 sample reactions were transformed into XL1 Blue cells. At least four colonies from each sample were selected and kept at -80 °C.

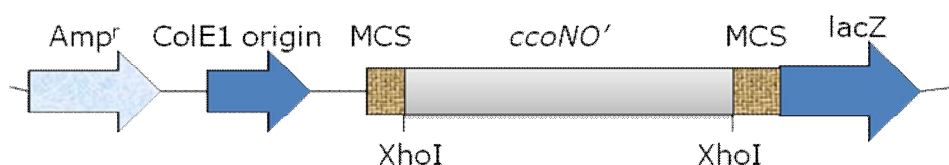


Figure 3. 1 Map of pMOZI, 5.7 kbp. The pMOZI was constructed by ligation of the 2.8-kbp *XhoI* fragment of pOX15 into the *XhoI* site of pBluescript SK+ DNA, 2.9 kbp [74].

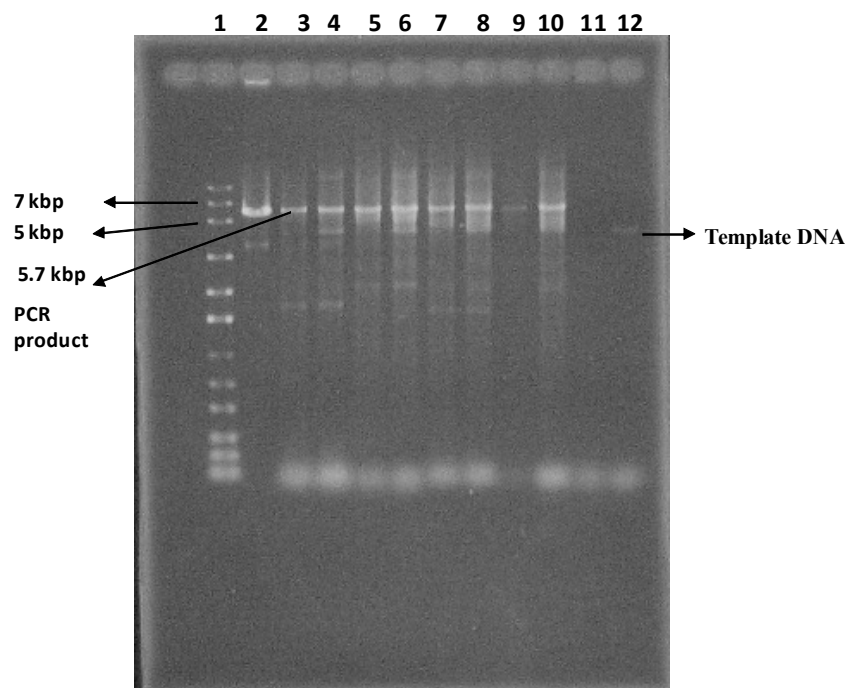


Figure 3. 2 pMOZID361, pMOZIE380 and pMOZIK387 PCR products (10 μ l) loaded on 1 % agarose gel. Line 1: marker; line 2: linear pMOZI DNA; line 3: pMOZID361 with *DpnI* sample reaction; line 4: pMOZID361 sample reaction; line 5: pMOZIE380 with *DpnI* sample reaction; line 6: pMOZIE380 sample reaction, line 7-9: pMOZIK387 with *DpnI* sample reactions, line 8-10: pMOZIK387 sample reactions and line 11-12 control reactions with and without *DpnI*.

3.2. The Construction of pMOZIII Vector DNA

To construct pOX15* expression vector DNAs, containing one of the three desired mutations, 2.8 kbp mutated fragment from pMOZI* must be replaced with that of pOX15. Replacement of 2.8 kbp fragment from pMOZI DNA directly with that pOX15 is neither possible nor economic. When pMOZI* DNA was digested with *XhoI* restriction enzyme which generates 2.8 and 2.9 kbp DNA fragments from pMOZI* DNA. Therefore, obtaining of 2.8 kbp fragments from pMOZI DNA is difficult. On the other hand, to replace 2.8 kbp DNA fragment from pMOZI with that of pOX15 DNA whole 2.8 kbp DNA fragments must be

sequenced. Sequencing of whole 2.8 kbp fragment from three different mutants is time consuming and not economic. Due to these reasons, pMOZIII vector was constructed.

To construct pMOZIII vector DNA, pMOZII [38] was digested with *Ppu2II*. Digested DNA samples were loaded on a 1% agarose gel and resulting fragments, 6.2 kbp and 0.6 kbp, were separated (Figure 3.4). 6.2 kbp DNA fragment was isolated from agarose gel and then recloned by ligation reaction at 22 °C for 2 hours. Ligation reaction mixture was transferred into CaCl₂ treated HB101 cells, *E.coli* strain. The transformants were selected based on kanamycin sensitivity. New clone was named as pMOZIII.

3.3. The Construction and Analysis of GK32/pOX15D361N Mutant

3.3.1. The Sequencing of pMOZID361N Mutant

Plasmid DNA from pMOZID361-1 transformants was sequenced using primer of F568 5'GGA CAA GCT GCG CAC CCT GCC GGT GAT CCG G-3' produced by Iontek Company, Istanbul. The sequence obtained from pMOZID361 plasmid DNA was compared with the sequence of the *cbb₃* oxidase from gene data bank. It was found that the sequence has a desired mutation (CTG) and there is no mutation other than desired one (Figure 3.3). The mutated codon is underlined. The pMOZ1D361 clone was named as pMOZID361N.

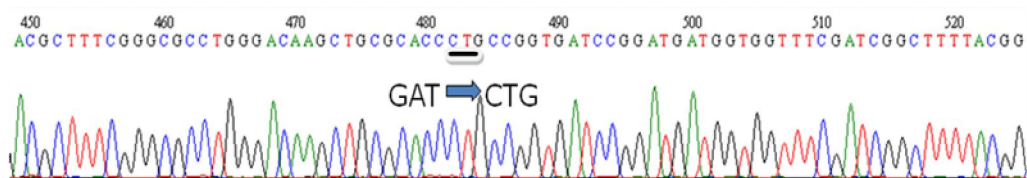


Figure 3. 3 The partial sequence of pMOZID361N indicating mutated codon.

3.3.2. The Construction of the Desired pMOZIID361N Mutant from the pMOZID361N

To replace 0.6 kbp fragment of pMOZIII with that of pMOZID361N, pMOZIII and pMOZID361N DNAs were digested with *ppu2II* (*BsAI*). The pMOZIII DNA was applied with shrimp alkaline phosphatase (SAP- Fermentas), to prevent self ligation of vector during the ligation reaction. The digested pMOZID361N and pMOZIII were loaded on 1% agarose gel and resulting fragments, 0.6 kbp, 1.1 kbp and 4.0 kbp fragments from pMOZID361N and 6.2 kbp fragment from pMOZIII were separated (Figure 4.4). Then linear 6.2 kbp pMOZIII vector and 0.6 kbp insert fragments from pMOZID361N were isolated from 1% agarose gel by using QIAGEN QIAquick® Gel Extraction Kits (Figure 3.5. A-B).

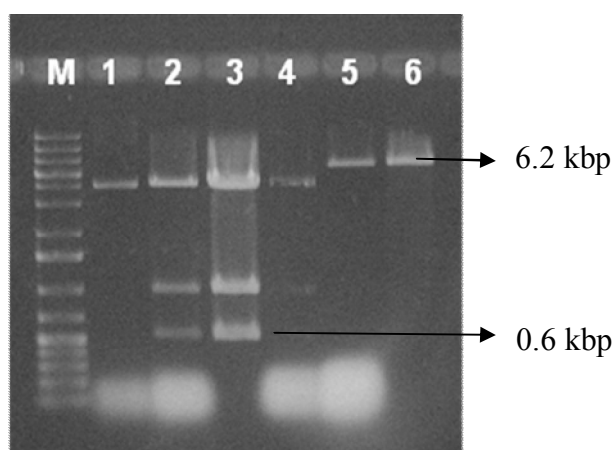


Figure 3. 4 *Ppu2II* (*BsAI*) digested vector and insert DNAs: (M) Marker, (1) Uncut pMOZI DNA, (2, 3) *Ppu2II* (*BsAI*) digested pMOZID361N resulting 0.6

0.6 kbp, 1.1 kbp and 4 kbp fragments, (4) Uncut pMOZIII, (5, 6) *ppu2II* (*Bsa*AI) digested linear pMOZIII DNAs.

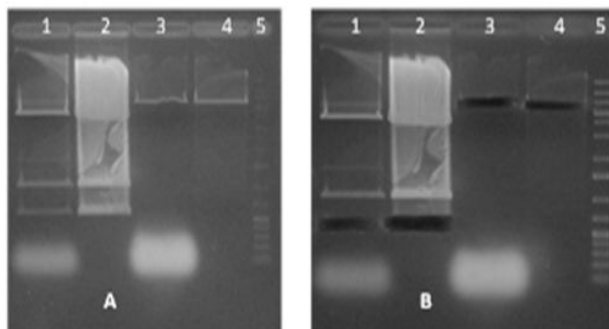


Figure 3. 5 Separation and isolation of 0.6 kbp insert and 6.2 kbp vector DNAs from 1 % agarose gel. Gel A: (Line 1, 2) Separation of 0.6 kbp, 1.1 kbp and 4 kbp fragments of pMOZID361. (Line 3, 4) Linearization of 6.2 kbp pMOZIII DNA. Gel B: (Line 1, 2) Isolation of 0.6 kbp insert DNA of pMOZID361, (Line 3, 4). Isolation of linear 6.2 kbp pMOZIII DNAs.

0.6 kbp mutated fragment was inserted into 6.2 kbp vector by incubating at 22 °C for 1 hour in the presence of PEG. Finally 10 µl ligation mix was transformed into 100 µl HB101 competent cells. The transformants were selected based on the size of the clone, 6.8 kbp.

Insert DNA could be inserted into the vector DNA in two orientations during ligation. To select correctly oriented mutant, DNAs from the four different transformants were digested with *Sal*I (Fermentas) restriction enzyme. This enzyme cut once vector and once insert resulting 1.5 kbp and 5.3 kbp fragments if insert is correct oriented and 1.1 and 5.7 kbp fragments if insert is wrong oriented (Figure 3.8). The new clone that has correct oriented insert was named as HB101/pMOZIID361N and stored at -80 °C.

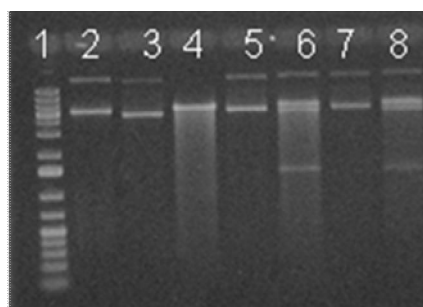


Figure 3. 6 0.6 kbp fragment orientation test by digestion of pMOZIID361N with *SalI*. (1) Marker, (2) Circular control pMOZII DNA, (3) Uncut pMOZIID361N/1, (4) *SalI* digested pMOZIID361N, (5) Uncut pMOZIIND361N/2, (6) *SalI* digested pMOZIID361N/2 with correct orientation resulting 1.5 kbp and 5.3 kbp fragments, (7) Uncut pMOZIID361N/3, (8) *SalI* digested pMOZIID361N/3 with correct orientation resulting 1.5 kbp and 5.3 kbp fragments.

3.3.3. The Construction of pOX15D361N Vector

To replace the 2.8 kbp fragment of pOX15 with the 2.8 kbp mutated insert that contains mutation on D361 site, pMOZIID361N was digested with *XhoI* (Fast Digest, Fermentas) at 37 °C for 45 min (Figure 3.7, line 6-7). At the same time, pOX12 DNA (12.5 kbp) was isolated from overnight culture by using Wizard® Plus SV Minipreps DNA Purification System (Promega) and digested with *XhoI* (Fast Digest, Fermentas) at 37 °C for 45 min and also dephosphorylated by shrimp alkaline protease (SAP- Fermentas) to prevent self ligation of the vector (Figure 4.9, line 3-4). The digested sample were loaded on 1% agarose gel and electrophoresed at 75 V for 1 hour. 2.8 kbp fragment from pMOZIID361N was isolated from agarose gel by using DNA Extraction Kit (#K0513) (Fermentas). 12.5 kbp linear pOX12 vector DNA were isolated from agarose gel by using High Pure PCR Product Purification Kit (Roche) (Figure 3.8, C-D). The 2.8 kbp mutated fragment was ligated into 12.5 kbp vector in total 20 µl ligation reaction occurred at 8°C for 16 hours. 5 µl ligation mixtures were transformed into 200 µl DH5α competent cells. The transformants were selected

on the basis of their size (15.3 kbp) from LB plate containing tetracycline antibiotic and new clone was named as DH5 α /pOX15D361 and stored at -80 °C.

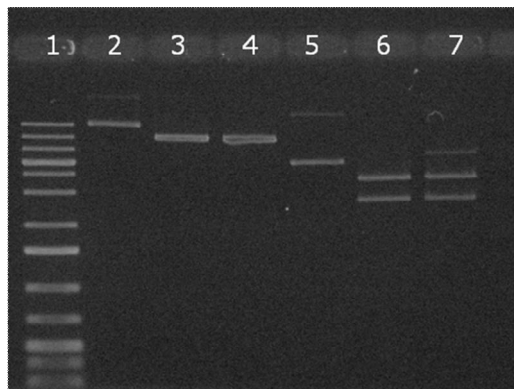


Figure 3. 7 The digestion of vector (pOX12) and insert (pMOZIID361N) DNAs with *Xho*I (Fast Digest) restriction enzyme. (1) Marker, (2) Uncut pOX12 DNA, (3, 4) Digested pOX12DNAs, (5) Uncut pMOZIID361N DNA, (6, 7) Digested pMOZIID361N DNAs.

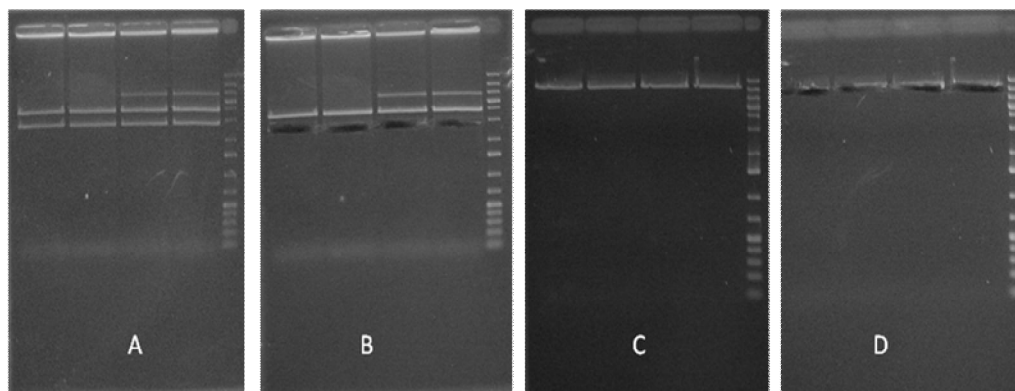


Figure 3. 8 The isolation of 2.8 kbp and 12.5 kbp fragments of *Xho*I digested pOX12 and pMOZIID361N DNAs from the 1% agarose gel. (A) Separation of 4 kbp and 2.8 kbp fragmens of *Xho*I digested pMOZIID361N DNA, (B) Isolation of 2.8 kbp fragment form the agarose gel. (C) Separation of linear 12.5 kbp fragment of *Xho*I digested pOX12 DNA, (D) Isolation of 12.5 kbp fragment from the agarose gel.

During ligation, 2.8 kbp fragments could be inserted into pOX12 vector in two orientations. In order to select the clone that has correct oriented insert, plasmid DNAs were isolated from two or more transformants then they were digested with *Hind*III (Fermentas) restriction enzyme which cuts the vector and the insert one time resulting either 3.6 kbp and 11.7 kbp fragments if insert is correct oriented, or 2.8 kbp and 12.5 kbp fragments if the insert is wrong oriented (Figure 3.9). The new clone that has correct oriented insert was kept at -80 °C and named as DH5 α /pOX15D361N.

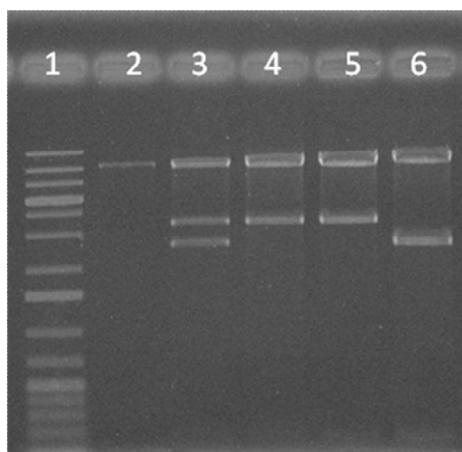


Figure 3. 9 2.8 kbp insert fragment orientation test by digesting of pOX15/D361N DNA with *Hind*III enzyme. (1) Marker, (2) 12.5 kbp linear pOX12 DNA, (3) *Hind*III digested pOX15D361N DNA with double insert, 3.6 kbp, 2.8 kbp and 11.7 kbp fragments, (4, 5) *Hind*III digested pOX15D361N DNAs with 3.6 kbp and 11.7 kbp fragments (correct oriented insert), (6) *Hind*III digested pOX15D361N DNA with 2.8 kbp and 12.5 kbp fragments (wrong oriented insert).

3.3.4. The Transformation of pOX15D361N into *R. capsulatus*

Triparental mating is a form of bacterial conjugation where a conjugative plasmid present in one bacterial strain assists the transfer of a mobilizable plasmid present in a second bacterial strain into a third bacterial strain. The

pOX15D361N plasmid DNA in DH5 α strain 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 resistance. GK32/pOX15D361N name was given to the selected transconjugants and kept at -80 °C for further analysis.

3.3.5. The NADI Analysis of the Cytochrome *cbb*₃ Oxidase Phenotype of GK32/pOX15D361N by NADI Test

NADI is a heme staining, which identify the mutation affecting on cytochrome *cbb*₃ oxidase phenotype by colony pigmentation, to test oxidase activity. NADI staining was performed for 30 seconds with whole cells of GK32/pOX15, as positive control, GK32, as negative control, and targeted mutants that had been grown under aerobic conditions. GK32/pOX15 was complemented mutant and gave NADI^{positive}, whereas GK32 was NADI^{negative} as expected. The desired mutant GK32/pOX15D361N was NADI^{positive}. NADI staining of GK32/pOX15D361N mutant indicated that asparagine mutation didn't effect enzyme activity (Fig. 3.10). Therefore, aspartate amino acid is found not to be a critical amino acid for enzyme activity even though they are conserved most of the subunit I of the *cbb*₃ type oxidases.



Figure 3. 10 NADH staining. (1) Positive control, GK32/pOX15 (NADH^{positive}), (2) Mutant GK32/pOX15D361N (NADH^{positive}), (3) Negative control, GK32 (NADH^{negative}).

3.4. The Construction and Analysis of GK32/pOX15E380Q Mutant

3.4.1. The Sequencing of pMOZIE380Q Mutant

0.6 kbp part of plasmid DNA from XL1-Blue/pMOZIE380Q transformant was sequenced using a primer F568 5' GGC ATG TCG ACC TTT **CAG** GGC CCG ATG ATG TCG -3' generated by Iontek Company, Istanbul. The sequence obtained from pMOZIE380-1 plasmid DNA was compared with the sequence of the *cbb₃* oxidase from gene data bank. It was found that the sequence has a desired mutation (CAG) changed from GAA (Fig. 3.11), and there was no mutation other than desired one. The mutated codon is underlined in figure 3.11. The pMOZIE380-1 clone was carrying glutamine amino acid instead of glutamic acid and was named XLI-Blue/ pMOZIE380Q.

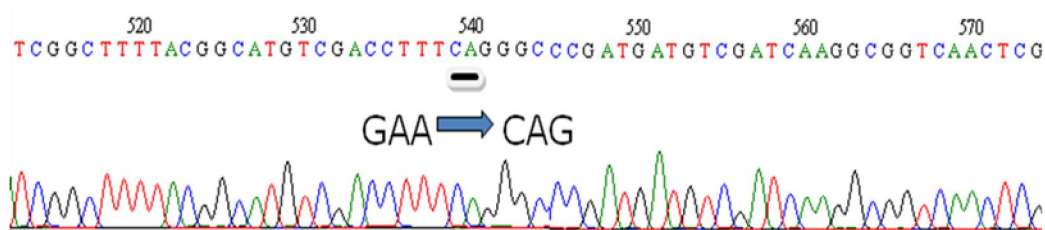


Figure 3. 11 The partial sequence of pMOZIE385Q indicating mutated codon.

3.4.2. The Construction of Desired pMOZIE380Q Mutant from pMOZIE380Q

The 0.6 kbp fragment of pMOZIE380Q DNA was replaced with that of the new 0.6 kbp DNA of pMOZIE380Q. First, vector, pMOZIII, and insert, pMOZIE380Q, DNAs were isolated by using GeneJET™ Plasmid Miniprep Kit (Fermentas cat. #: K0502). Then they were digested with *ppu2II* (*BsaI*) restriction enzyme was used. The *ppu2II* digested pMOZIII DNA was dephosphorylated with shrimp alkaline phosphatase, (SAP- Fermentas), to prevent self ligation. The *Ppu2II* digested pMOZIII and pMOZIE380Q DNA samples were loaded on 1% agarose gel and resulting fragments, 0.6 kbp, 1.1 kbp and 4.0 kbp fragments from pMOZIE380Q (Figure 3.12, line 5-6) and linear 6.2 kbp from pMOZIII were separated (Figure 3.12, line 2-3). Then 6.2 kbp from pMOZIII vector and 0.6 kbp insert fragments from pMOZIE380Q were isolated from 1% agarose gel by using QIAGEN QIAquick® Gel Extraction Kits (Figure 3.13). 0.6 kbp mutated fragment was inserted into 6.2 kbp vector by incubating at 22 °C for two hours in the presence of PEG. Finally 10 µl ligation mix was transformed into 100 µl HB101 competent cells. The transformants were selected based on the size of the clone, 6.8 kbp in size.

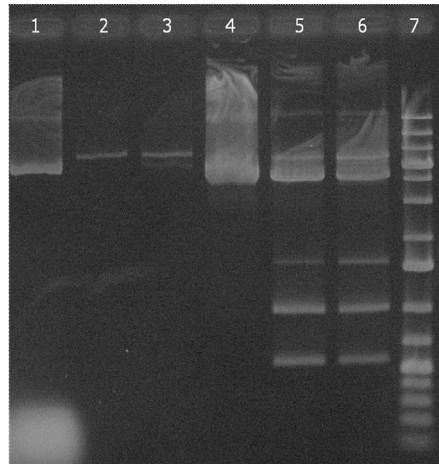


Figure 3. 12 *Ppu2II* (*BsAI*) digested vector and insert DNAs: (1) Uncut pMOZIII, (2, 3) *Ppu2II* (*BsAI*) digested linear pMOZIII DNAs, (4) Uncut pMOZI DNA, (5, 6) *Ppu2II* (*BsAI*) digested pMOZIE380Q resulting 0.6 kbp, 1.1 kbp and 4 kbp fragments, (7) Marker.

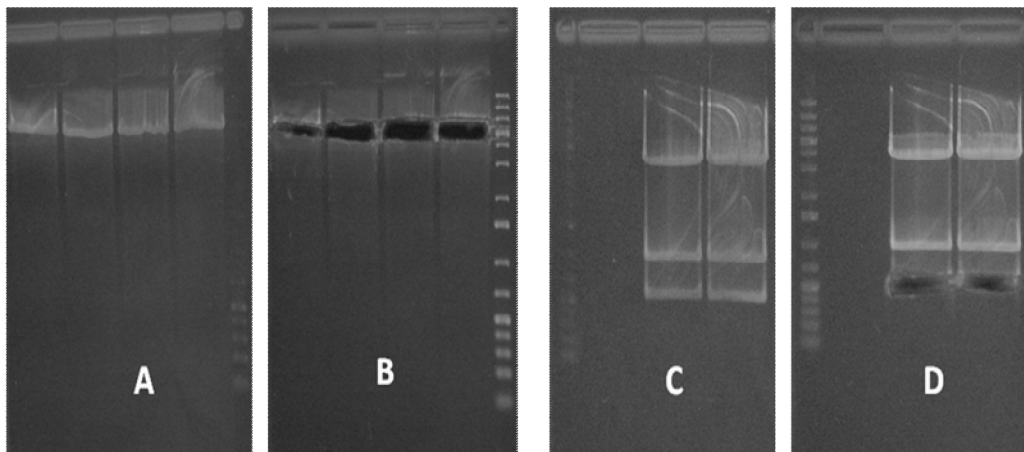


Figure 3. 13 The separation and isolation of *Ppu2II* (*BsAI*) digested 0.6 kbp insert and 6.2 kbp vector DNAs from 1 % agarose gel. (A) Linearization of 6.2 kbp pMOZIII DNA. (B) Isolation of linear pMOZIII DNA. (C) Separation of 0.6 kbp, 1.1 kbp and 4 kbp fragments of pMOZIE380Q, (D) Isolation of 0.6 kbp insert DNA of pMOZIE380Q.

To select correct oriented mutant, DNAs were digested with *SalI* (Fermentas) restriction enzyme. The clone that has right oriented insert was detected. This enzyme cut once vector and once insert resulting 1.5 kbp and 5.3 kbp fragments if insert is correct oriented and 1.1 and 5.7 kbp fragments if insert is wrong oriented (Figure 3.14). The new clone that has correct oriented mutated

insert was named as HB101/pMOZIIIE380Q and stored at -80 °C for the next process.

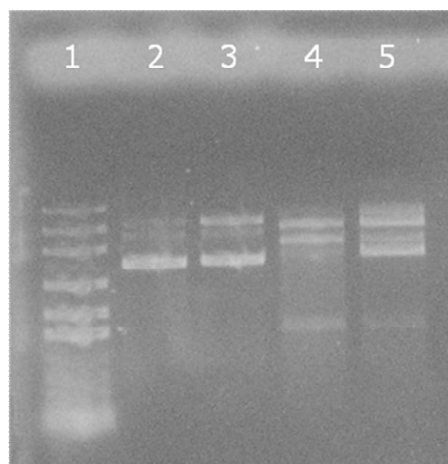


Figure 3. 14 0.6 kbp fragment orientation test by digestion of pMOZIIIE380Q with *SalI*. (1) Marker, (2) Circular pMOZII DNA, (3) Uncut pMOZIIIE380Q, (4) *SalI* digested pMOZIIIE380Q with correct orientation resulting 1.5 kbp and 5.3 kbp fragments, (5) *SalI* digested pMOZIIIE380Q with correct orientation resulting 1.5 kbp and 5.3 kbp fragments.

3.4.3. The Construction of pOX15E380Q Expression Vector

To replace the 2.8 kbp fragment of pOX15 with the 2.8 kbp mutated insert DNA that contains E380Q mutation, pMOZIIIE380Q was digested with *XhoI* (Fast Digest, Fermentas) restriction enzyme (Figure 4.15, B) for 30 min at 37 °C. At the same time, pOX12 DNA (12.5 kbp) was isolated from overnight culture by using Wizard® Plus SV Minipreps DNA Purification System (Promega) and digested with *XhoI* (Fast Digest, Fermentas) for 45 min at 37 °C and also dephosphorylated by shrimp alkaline protease (SAP- Fermentas) to prevent self ligation of the vector (Figure 3.15, A). Digested sample were loaded on 1% agarose gel and electrophoresed at 75 V for 1 hour. 2.8 kbp and 12,5 kbp fragments were obtained by digestion of pMOZIIIE380Q and pOX12 with *XhoI* and respectively the 2.8 kbp fragment was isolated from agarose gel (Figure 3.16- A, B) by using DNA Extraction Kit (#K0513- Fermentas), 12.5 kbp fragment

from pOX12 were isolated from agarose gel (Figure 3.16-C, D) by using QIAGEN QIAquick[®] Gel Extraction Kit. During the construction of pOX15E380Q, the 2.8 kbp mutated fragment was ligated into 12.5 kbp vector ligation process in 20 µl ligation reaction volume at 8°C for 16 hours. 5 µl ligation mixtures were transformed into 200 µl DH5α competent cells. Transformants were selected on the basis of their size (15.3 kbp) from LB plate containing tetracycline antibiotic (Figure 3.17) and new clone was named as DH5α/pOX15E380Q and stored at -80 °C.

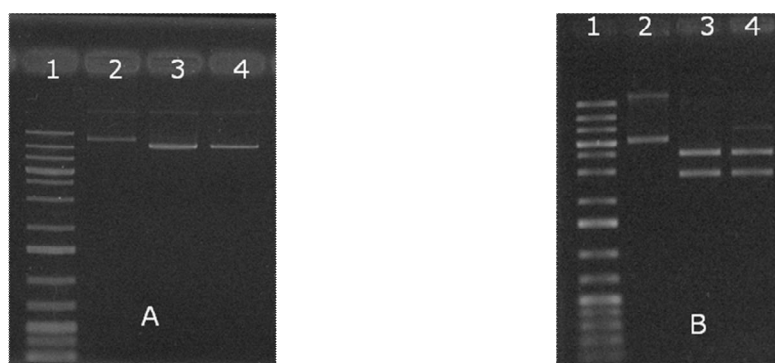


Figure 3. 15 The digestion of pOX12 and pMOZIIIE380Q DNAs with *Xho*I. Gel A-(1) Marker, (2) Uncut pOX12 DNA, (3, 4) *Xho*I (fast digestion) digested linear 12.5 kbp pOX12 DNAs. Gel B-(1) Marker, (2) Uncut pMOZII DNA for control of digestion, (3, 4) *Xho*I (fast digestion) digested pMOZIIIE380Q DNAs resulting 4.0 kbp and 2.8 kbp fragments.

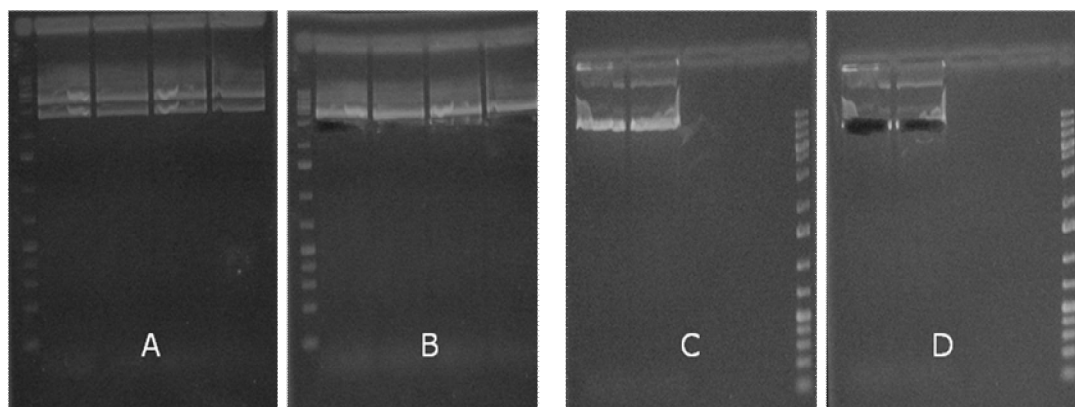


Figure 3. 16 Isolation of 2.8 kbp and 12.5 kbp fragments of *Xho*I digested pOX12 and pMOZIIIE380Q DNAs from the 1% agarose gel. (A) Separation of 4

kbp and 2.8 kbp fragments of *Xho*I digested pMOZIIIE380Q DNA, (B) Isolation of 2.8 kbp fragment from the agarose gel. (C) Linear 12.5 kbp fragment of *Xho*I digested pOX12 DNA, (D) Isolation of 12.5 kbp fragment from the agarose gel.

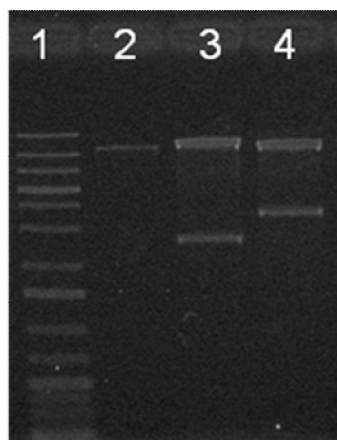


Figure 3. 17 *Hind*III digestion of obtained clones, (1) Marker, (2) pOX12-linear DNA, (3) pOX15E380Q-1/ *Hind*III with wrong orientation, (4) pOX15E380Q-2/ *Hind*III with right orientation.

3.4.4. The Transformation of pOX15E380Q into *R. capsulatus*

Three parental mating was used to transfer the pOX15E380Q mutant plasmid into *R. capsulatus* strain, GK32 in the presence of HB101/pRK2013 cells as a helper. Transconjugants were selected based on tetracycline resistance. Four transconjugants were selected and kept at -80 °C for further analysis, NADI staining.

3.4.5. The Analysis of the Cytochrome *cbb*₃ Oxidase Phenotype of GK32/pOX15E380Q by NADI Test

Transconjugants were tested with NADI staining for their oxidase phenotype in one MedA plate containing positive control (GK32/pOX15), negative control (GK32) and the desired mutant GK32/pOX15E380Q. Whereas

NADI staining of GK32/pOX15 was NADI^{positive}, GK32 was NADI^{negative} as expected. On the other hand, the desired mutant GK32/pOX15E380Q was NADI^{negative}. The GK32/pOX15E380Q colonies did not turn blue when it was compared with wild type which turns in blue within 30 seconds (Figure 3.18). NADI staining phenotype of this mutant indicates that the glutamate at position 380 must be a critical amino acid for enzyme activity.

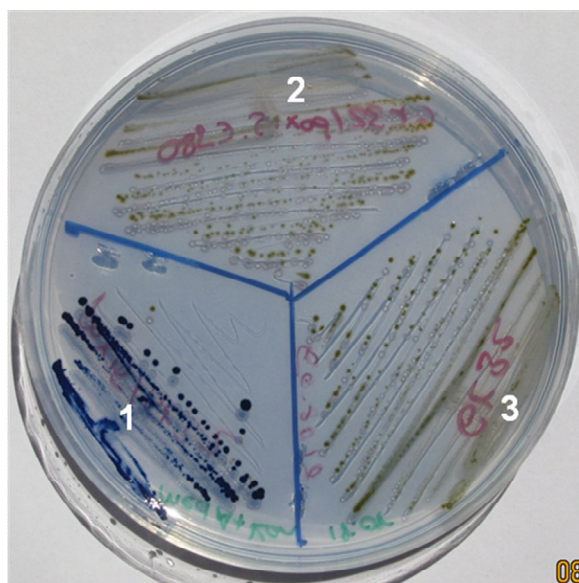


Figure 3. 18 NADI-stained GK32/pOX15E380Q plate. (1) Positive control, GK32/pOX15 (NADI^{positive}), (2) Mutant GK32/pOX15E380Q (NADI^{negative}), (3) Negative control, GK32 (NADI^{negative}).

3.5. The Construction and Analysis of GK32/pOX15K387M Mutant

3.5.1. The Sequencing of pMOZIK387M Mutant

The 0.6 kbp fragment of plasmid DNA from pMOZIK387-1 transformant was sequenced (Fig. 3.19) using primer F568 5'-CCG ATG ATG TCG ATC ATG GCG GTC AAC TCG C-3' generated by Iontek Company, Istanbul. Sequence obtained from pMOZIK387-1 plasmid DNA was compared with the

sequence of the *cbb₃* oxidase from gene data bank. It was found that the sequence has a desired mutation (ATG) changed from AAG, and there is no mutation other than desired one. The mutated codon was underlined in Figure 3.19. The pMOZIK387-1 clone containing methionine amino acid instead of lysine, was named pMOZIK387M.

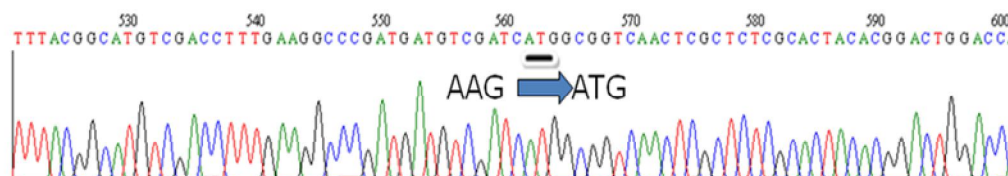


Figure 3. 19 The partial sequence of pMOZIK387M indicating mutated codon.

3.5.2 The Construction of the Desired pMOZIK387M Mutant from the pMOZIK387M

0.6 kbp mutated DNA fragment was inserted into pMOZIII to obtain pMOZIK387M. At the first step, the vector and insert DNAs were isolated by using GeneJET™ Plasmid Miniprep Kit (Fermentas cat. #: K0502) and both DNAs were digested with *Ppu2II* (*BsAI*- Fermentas) restriction enzyme. The pMOZIII DNA was dephosphorylated with shrimp alkaline phosphatase, (SAP- Fermentas), to prevent self ligation. pMOZIK387M and pMOZIII DNA samples were loaded on 1% agarose gel and resulting fragments, 0.6 kbp, 1.1 kbp and 4.0 kbp fragments from pMOZIK387M (Figure 3.20-A) and 6.2 kbp from pMOZIII were separated (Figure 3.20-B). Then 6.2 kbp vector and 0.6 kbp insert fragments were isolated from 1% agarose gel by using QIAGEN QIAquick® Gel Extraction Kits (Figure 3.21) and then 0.6 kbp mutated fragment was inserted into 6.2 kbp vector by incubating at 22 °C for two hours in the presence of PEG. Finally 10 µl ligation mix was transformed into 100 µl HB101 competent cells. Transformants were selected based on the size of the clone, 6.8 kbp.

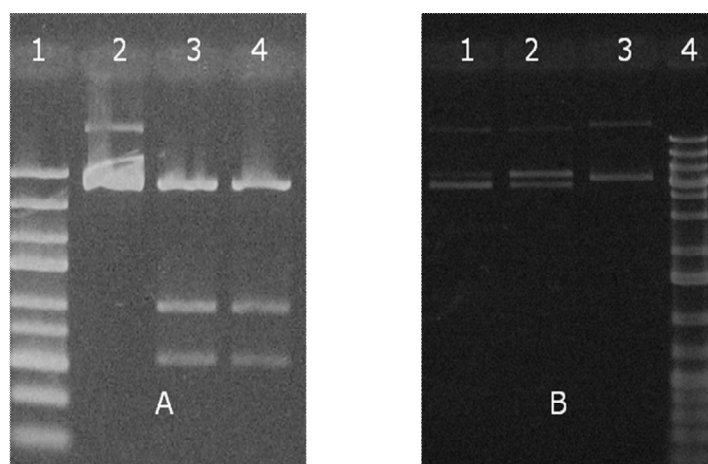


Figure 3. 20 *Ppu2II* (*BsaI*) digested vector and insert DNAs. Gel A- (1) Marker, (2) Uncut pMOZI DNA, (3, 4) *Ppu2II* digested pMOZIK387M resulting 0.6 kbp, 1.1 kbp and 4 kbp fragments, Gel B- (1, 2) *Ppu2II* digested linear pMOZIII DNAs, (3) Uncut pMOZIII, (4) Marker.

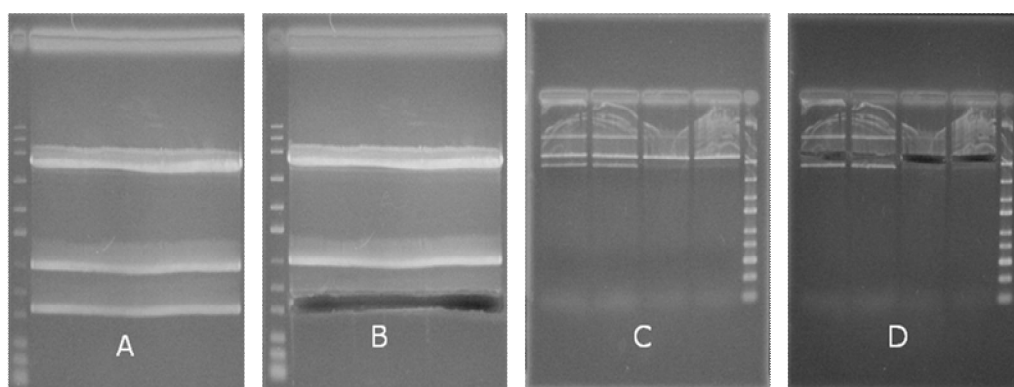


Figure 3. 21 The separation and isolation of *Ppu2II* digested 0.6 kbp insert and 6.2 kbp vector DNAs from 1 % agarose gel. (A) Separation of 0.6 kbp, 1.1 kbp and 4 kbp fragments of pMOZIK387M, (B) Isolation of 0.6 kbp insert DNA of pMOZIK387M, (C) Linearization of 6.2 kbp pMOZIII DNA, and (D) Isolation of linear pMOZIII DNA.

To select the correctly oriented mutant, DNAs were digested with *SalI* (Fermentas) restriction enzyme. The new clone that contains correct oriented mutated insert (Figure 3.22) was named as HB101/pMOZIIK387M and stored at - 80 °C.

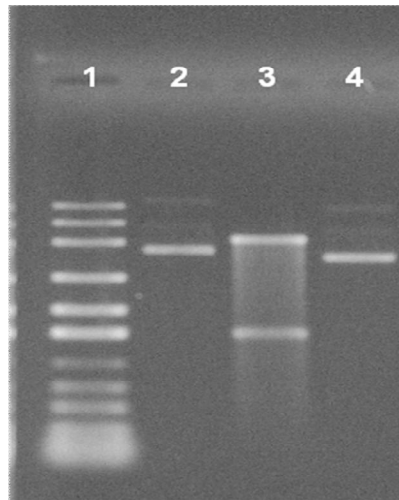


Figure 3. 22 0.6 kbp fragment orientation test by digestion of pMOZIIK387M with *SalI*. (1) Marker, (2) Uncut pMOZIII, (3) *SalI* digested pMOZIIK387M with correct orientation resulting 1.5 kbp and 5.3 kbp fragments, (4) Uncut pMOZII as control.

3.5.3. The Construction of pOX15K387M

To replace the 2.8 kbp fragment of pOX15 with the 2.8 kbp DNA fragment containing mutation at 387 position of pMOZIIK387M, pMOZIIK387M was digested with *XhoI* (Fermentas) at 37 °C for 3 hours (Figure 4.23-B). At the same time, pOX12 DNA (12.5 kbp) was isolated from overnight culture by using Wizard® Plus SV Minipreps DNA Purification System (Promega) and digested with *XhoI* (Fast Digest, Fermentas) at 37 °C for 45 min and also dephosphorylated by shrimp alkaline protease (SAP- Fermentas) to prevent self ligation (Figure 3.23-A). The digested samples were loaded on 1% agarose gel and electrophoresed at 75 V for 1 hour. The 2.8 kbp obtained fragment from pMOZIIK387M was isolated from the agarose gel (Figure 3.24 –A and B) by using DNA Extraction Kit (#K0513- Fermentas) to use insert DNA. 12.5 kbp fragment from pOX12 were isolated from the agarose gel (Figure 3.24 – C and D) by using High Pure PCR Product Purification Kit (Roche). The 2.8 kbp

insert DNA, containing desired mutation, was ligated into 12.5 kbp vector by ligation reaction in total 20 μ l ligation reaction volume at 8°C for 16 hours. 5 μ l ligation mixtures were transformed into 200 μ l DH5 α competent cells. The transformants were selected on the basis of their size (15.3 kbp) from LB plate containing tetracycline antibiotic and new clone was named as DH5 α /pOX15K387M (Figure 3.25) and stored at -80 °C for further analysis.

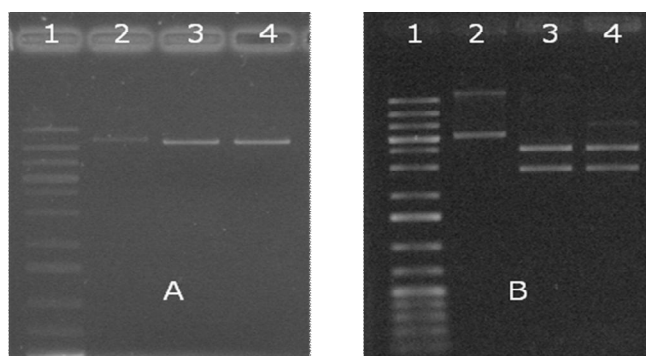


Figure 3. 23 The digestion to obtain of 12.5 kbp linear vector DNA and 2.8 kbp insert DNA. Gel A- (1) Marker, (2) uncut pOX12 DNA, (3, 4) Digested linear 12.5 kbp pOX12 DNAs. Gel B- (1) Marker, (2) Uncut pMOZIIK387M DNA, (3, 4) Digested pMOZIIK387M DNAs resulting 4.0 kbp and 2.8 kbp fragments.

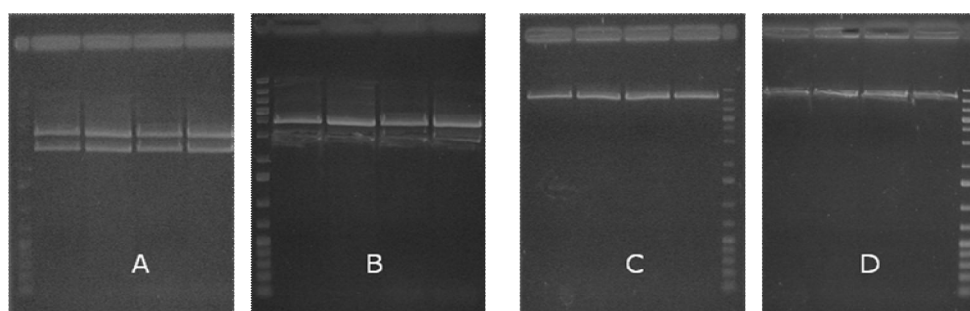


Figure 3. 24 The isolations of 2.8 kbp and 12.5 kbp fragments from digested pOX12 and pMOZIIK387M DNAs from the 1% agarose gel. (A) Separation of 4 kbp and 2.8 kbp fragments of digested pMOZIIK387M DNA, (B) Isolation of 2.8 kbp fragment from the agarose gel. (C) Linear 12.5 kbp fragment of digested pOX12 DNA, (D) Isolation of 12.5 kbp fragment from the agarose gel.

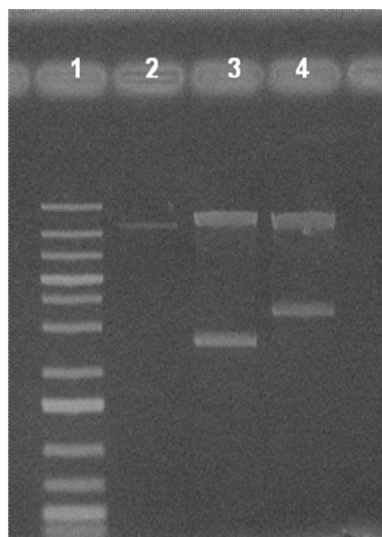


Figure 3. 25 *Hind*III digestion of pOX15K387M DNA. (1) Marker, (2) Linear pOX12, (3) pOX15K387M/*Hind*III with not correct orientation, (4) pOX15K387M/*Hind*III with correct orientation.

3.5.4. The Transformation of pOX15K387M into *R. capsulatus*

Three parental mating was used to transfer the pOX15K387M mutant plasmid into *R. capsulatus* strain, GK32, in the precence of HB101/pRK2013 helper cells. Transconjugants were selected based on tetracycline resistance. Four transconjugants were selected and kept at -80 °C for further analysis.

3.5.5. The Analysis of the Cytochrome *cbb₃* Oxidase Phenotype of GK32/pOX15 K387M by NADI Test

The transconjugants of GK32/pOX15K387M were tested with NADI staining for their oxidase phenotype in MedA plate in the presence of negative control (GK32) and positive control (GK32/pOX15). NADI staining indicated that GK32/pOX15 was NADI^{positive} and GK32 was NADI^{negative} as expected. On the other hand, the desired mutant GK32/pOX15K387M was NADI^{positive}. The

GK32/pOX15K387M colonies did not turn blue when it was compared with wild type which turns in blue within 30 seconds (Figure 3.26). NADI staining phenotype of this mutant indicates that the lysine at position 387 could not be a critical amino acid for enzyme activity.

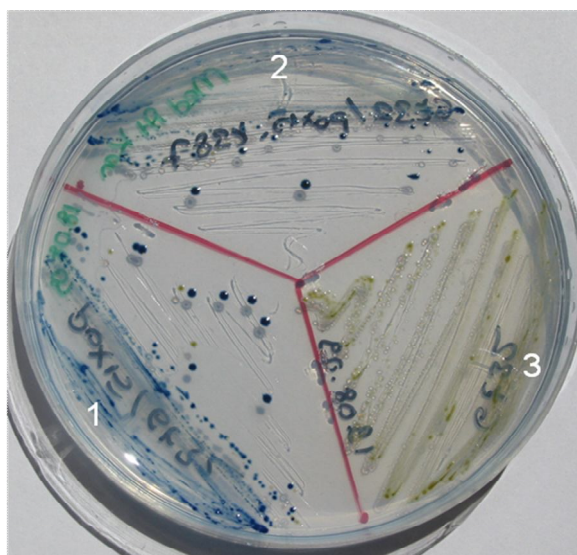


Figure 3. 26 NADI staining of GK31/pOX15K387M. (1) Positive control, GK32/pOX15 (NADI^{positive}) (2) Mutant GK32/pOX15K387M (NADI^{positive}), (3) Negative control, GK32 (NADI^{negative}).

CHAPTER 4

DISCUSSION

The heme-copper oxidases are large multimeric membrane proteins, characterized by their binuclear active site. These enzymes are key metabolic enzymes in aerobic prokaryotes and mitochondria. They reduce molecular oxygen with electrons from catabolic reactions and generate an electrochemical gradient, which in turn drives synthesis of ATP to be used in anabolic reactions. The active site of these enzymes is buried deep in the helical bundle and consists of a high-spin heme (A, B, or O type) and a copper atom (Cu_B), between which the substrate oxygen binds. Cu_B is ligated by three invariant histidines, one of which is covalently bound to a unique tyrosine, which participates in the catalytic reaction [40]. The members of the heme-copper superfamily are divided into three distinct families of oxygen reductases: the A-, B-, and C-families [4]. The variation in heme-copper oxygen reductases is due to the differences in the active site and proton channels. The A- and B-families need either an *o* or *a* heme in the active site (the hydroxyl group on the tail is part of the K-channel), whereas the C-family utilizes *b* heme in the active site (they use a conserved tyrosine in place of the tail) [15].

The A-family of oxygen reductases is the largest and most studied group of enzymes in the heme-copper superfamily. Several X-ray structures and site-directed mutagenesis along with biophysical methods have been extensively used to elucidate the structural and mechanistic properties of this family [19]. Two proton-conducting input channels, the D- and K-channels, are well defined by

both X-ray crystallography and site-directed mutagenesis of prokaryotic members of the family [79]. In the A-type oxygen reductases, the K-channel leads from a surface-exposed glutamate at the cytoplasmic surface to the active site tyrosine, which is cross-linked to a histidine [69]. Besides the active site tyrosine, K362 residue (numbering of the *R. sphaeroides* *aa*₃-type oxidase) is conserved in the A-family of enzymes. Similarly, there is a pattern of highly conserved residues in the D-channel in the A-family of oxygen reductases, E286. These two channels are used to provide protons from the bacterial cytoplasm to the active site for chemistry and for proton pumping. Previous data demonstrated that aspartate is found to be a critical amino acid for proton pumping activity in both *R. sphaeroides* cytochrome *aa*₃ [24] and *E. coli* cytochrome *bo*₃ [22]. Some mutations within the D-channel can eliminate proton pumping [80,81] whereas other mutations completely block all catalytic function [82].

K- and D- proton conduction residues which form the proton channels in the A-family are not conserved in the C-family of oxygen reductases at the same position within A-family and nothing is known about proton channels in the *cbb*₃ oxidases. E380 in helix IX is the only carboxylic amino acid, conserved among the *cbb*₃ enzymes, that is predicted to reside within the membrane domain at (or below) the level of the heme groups. Some recent structural data argued that conserved glutamate E383 (*R. sphaeroides* *cbb*₃ oxidase numbering) [40] and D364 on the cytoplasmic side in helix IX [19] might be involved in proton pumping. Supporting this idea, E383 was mutated to glutamine resulting in complete loss of activity [40, 45]. To understand the minimal common properties

of the superfamily, and to learn about *cbb*₃-type oxidases specifically, we have aligned a wide set of heme-copper oxidase sequences of the catalytic subunit of the *cbb*₃ oxidase from *Rhodobacter capsulatus*. We conclude that with regard to the active site surroundings, the *cbb*₃ oxidases greatly resemble the structurally known oxidases, while major differences are conserved residues position. The sequence analysis indicated that D361, E380 and K387 residues are conserved with in the *cbb*₃ oxidase in helix IX instead of helix VII. There fore conserved amino acids, D361, E280 and K387, were substituted for glutamine, leucine and methionine respectively. Our E380Q mutation completely inactivate *cbb*₃ oxidase and the result supports the finding of [17, 45] but controversy of [19], which claimed that there are no conserved hydrophilic residues in the C- type oxidases that could form either a channel analogous to the D- channel or an alternative channel from the cytoplasmic surface. The results of D361N and K387M mutants showed that these two conserved residues thought to be K- and D-channels have a wild-type enzyme activity. This is the first mutagenesis report on the conserved amino acids thought to be alternative proton pumping channels in *cbb*₃-type oxidase.

In conclusion, the combination of existing structural and sequence data on heme-copper oxidases has allowed us to construct models of the divergent members of the group, the *cbb*₃ oxidases, and to locate several functionally important residues. The active site structure can be very different in the subfamilies of the heme-copper oxidases, and well conserved within individual subfamilies, even though the overall function is shared. These results strongly support the proposal that the C-type oxygen reductases have only one proton input channel and that the K-channel is responsible for the uptake of all protons,

both for catalysis and pumping. Although the principles behind the mechanism of proton pumping are very likely to be the same, it is evident that the functions of residues in the D-channel is shown to be critical in the A-family of oxygen reductases, which must be performed differently in the C-family of oxygen reductases [19]. The diversity may reflect the optimization of different terminal oxidases for specific environmental requirements [50].

Future Work

For the future aspects, to determine the effects of the mutants, Asp361Asn, Glu380Gln and Lys387Met, on the activity, assembly and the function of the cytochrome *cbb*₃-type oxidase in *R. capsulatus* chromatophore membrane proteins will be isolated. The isolated chromatophore membrane proteins will be used for the determination of the oxygen consumption rate by using Clark-Type Oxygen electrode. Obtaining of membrane protein SDS-PAGE will help us to show differentiation by visualization and the effects of mutations can be proved as protein level by quantitatively.

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APPENDICES

APPENDIX A

1-Bacterial Growth Media and Antibiotics

Liquid LB (Luria Bertani) Medium

Contents	For 1lt
Bacto tryptone (Merck)	10 g
Yeast extract (Merck)	5 g
NaCl (Merck)	10 g

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

LB plate

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

Sistrom's Minimal Medium (Med A)

Contents	For 1lt
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 lt and autoclaved. 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

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

A) Microelements Solution

Contents	For 1lt
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 1lt dH₂O, autoclaved and stored at +4°C.

B) Macro elements Solution (Solution C)

Contents	For 1lt
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 lt with dH₂O, autoclaved and stored at +4°C.

C) Vitamins (100X)

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

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

SOB

Contents	For 1lt
Bacto tryptone (Merck)(2 %)	20 g
Yeast extract (Merck)(0.5 %)	5 g
NaCl (Merck)(10 mM)	0.584 g
KCl (Sigma)(2.5 mM)	0.188 g
MgCl ₂ .6H ₂ O (Sigma)(10 mM)	2.032 g
MgSO ₄ .7H ₂ O (Sigma)(10 mM)	2.464 g

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

SOC

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

APPENDIX B

Buffers and Antibiotics

Ampicillin

For 10 ml, 25 mg/ml Ampicillin stock solution, 0.25 g Ampicillin Sodium Salt (Sigma) was dissolved in 10 ml dH₂O and sterilized by using 0.22µm filter and stored at -20 °C.

Tetracycline

For 40 ml, 1.25 mg/ml Tetracycline stock solution, 0.05 g Tetracycline (Sigma) was dissolved in 40 ml 50 % (v/v) ethanol/dH₂O and sterilized by using 0.22µm filter and stored in dark at -20 °C.

Kanamycin

For 10 ml, 50 mg/ml Kanamycin stock solution, 0.05 g Kanamycin (Sigma) was dissolved in 10 ml dH₂O and sterilized by using 0.22µm filter and stored at -20 °C.

Chloramphenicol

For 10 ml, 50 mg/ml Chloramphenicol stock solution, 0.05 g Chloramphenicol (Sigma) was dissolved in 10 ml dH₂O and sterilized by using 0.22µm filter and stored at -20 °C.

Transformation Buffer (TB)

Contents	For 1lt
HEPES (Sigma)(10 mM)	0.960 g
CaCl ₂ .H ₂ O (Sigma)(15 mM)	0.880 g
KCl (Sigma)(250 mM)	7.456 g
MnCl ₂ .2H ₂ O (Sigma)(55mM)	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 lt with dH₂O and filter sterilized by using 0.22µm filter and stored at 4°C.

TAE Buffer (50X)

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

EDTA (Ethylenediamine-tetraacetic acid) (0.5 M)

For 250 ml EDTA solution, 46.5 g EDTA is dissolved in 150 ml dH₂O, and pH is adjusted with NaOH to 8,00. The volume is completed to 250 ml with H₂O and autoclaved.

TE

For 100 ml

Tris Base (1M, pH: 7.50) 1 ml

EDTA.2H₂O (0.5 M, pH: 8) 200 µl

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

APPENDIX C-CHEMICALS

Acetic acid (Merck, 100056)

Agar (Difco, 0140-01)

Agarose (Research organics 1170A) cat number 9012-36-6

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

Ampicillin (Sigma, A-0166)

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

Bacto peptone (Difco, 0118-17-0)

Bacto tryptone (Difco, 0123-17-3)

DMPD [N, N-Dimethyl -p-Phenylene -Diamine] (Sigma, D-5004)

N, N'-Methylene-bis-Acrylamide (Sigma, M-7256)

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

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

Glycerol, Electrophoresis grade (Sigma G-8773)

L-Glutamic acid (Sigma, G-1251)

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)

α -Naphthol (Sigma, N-1000)

Phenol (Sigma, P-4557)

Phenol (Merck, 100995)

Sodium acetate (Merck, 6268.1000)

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

Tetracycline Hydrochloride, Crystalline (Sigma, T-33839)

Tris Base (Trizma base, J.T.Baker 77-86-1)

Tricine (Electrophoresis Grade) Sigma T-7911

Yeast Extract (Difco, 0127-17-9).

APPENDIX D- ENZYMES

1-RESTRICTION ENDONUCLEASES

*Bsa*AI (ppu21I) (New England Biolabs): cat. # R0531S

*Sac*I (MBI): cat. # ER1131

*Dpn*I (MBI): cat. # ER1701

*Hind*III (MBI): cat. # ER0501

*Sal*I (MBI): cat. # ER0641

*Xho*I (MBI): cat. # ER0691

*Xho*I (Fast Digestion) (MBI): cat. # ER0691

2-POLYMERASES

Pfu DNA polymerase (MBI): cat. # EP0501

3-LIGASES

T4 DNA Ligase (MBI): cat. # EL0331

4-PHOSPHATASES

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

dNTP Set

dNTP (MBI): cat. # R0181

APPENDIX E- EQUIPMENTS USED IN THIS STUDY

PCR, Techne, TC 3000

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

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

Shaker- Heidolph Unimax 2010

Electrophoresis Systems

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, Finnpiette

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 Transalluminator (UVP)

Imaging system (UVP Photo Doc-It™)

Vortex (yellow^{line} TTS2)

Water Purification System (Human corporation)
