2018 년 2 월

석사 학위 논문

The essential role of small protein CydX for the cytochrome *bd* oxidase activity in *Salmonella enterica* Serovar Typhimurium

조선대학교 대학원

치의생명공학과

KIEU MINH DUC

The essential role of small protein CydX for the cytochrome *bd* oxidase activity in *Salmonella enterica* Serovar Typhimurium

살모넬라의 cytochrome *bd* oxidase 활성에 필수적인 작은 단백질 CydX 의 역할

2018 년 2 월 23 일

조선대학교 대학원

치의생명공학과

KIEU MINH DUC

The essential role of small protein CydX for the cytochrome *bd* oxidase activity in *Salmonella enterica* Serovar Typhimurium

살모넬라의 cytochrome *bd* oxidase 활성에 필수적인 작은 단백질 CydX 의 역할

지도교수 방 일 수

이 논문을 이학석사학위 신청 논문으로 제출함

2017 년 10 월

조선대학교 대학원

치의생명공학과

KIEU MINH DUC

KIEU MINH DUC 의 석사학위논문을 인준함

위원장 조선대학교 교 수 국 중 기 (인)

위 원 조선대학교 교 수 안 상 건 (인)

위 원 조선대학교 교 수 방 일 수 (인)

2017 년 11월

조선대학교 대학원

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

Amp^r	Ampicillin resistant phenotype
BME	ß-mercaptoethanol
Cm^r	Chloramphenicol resistant phenotype
DTT	Dithiothreitol
ETC	Electron transport chain
LB	Luria-Bertani broth
HCOs	Heme-copper oxidases
TMPD	1% N,N,N',N'-tetramethyl-p-phenylenediamine
sNO	spermine NONOate
UT	Untreated
WT	Wild-type
SD	Standard deviation

ABSTRACT

The essential role of small protein CydX for the cytochrome bd oxidase activity in *Salmonella enterica* Serovar Typhimurium

Kieu Minh Duc

Advisor: Prof. Bang, Iel-Soo , PhD

Department of Biodental Engineering,

Graduate School of Chosun University

Cytochrome bd oxidase, a terminal oxidase enzyme complex that is necessary for the survival of prokaryotes under less oxic conditions. This enzyme complex has 3 subunits including cydA, cydB and small protein ybgT renamed as CydX. Although the role of this small protein CydX is reported in *Escherichia coli* and other species but still not clearly known in prokaryotic pathogen, *Salmonella* Typhimurium. In this current study, we confirmed the previous finding that CydX is a part of the enzyme complex and is required for oxidase activity and assembly in *S.* Typhimurium as in *Escherichia coli.* Furthermore, we also confirmed that the reduced cytochrome bd oxidase is sensitive to ß-mercaptoethanol (reducing agent) in both aerobic and low oxygen condition. We propose that the sensitivity of ß-mercaptoethanol is due to inhibition of bacterial respiration and not disulfide bond breakage. The cytochrome bd oxidase protects bacterial respiratory activity while ß-mercaptoethanol targets to heme-copper of cytochrome bo oxidase. It suggested that cytochrome bd oxidase can be a potential drug target for antimicrobial therapy in near future.

I. INTRODUCTION

Salmonella enterica is a gram-negative bacteria as a member of the enterobacteriaceae family pathogenic. It has genetic structure closely related to *Escherichia coli*. Animals and human can uptake *Salmonella enterica* serovars by contaminated food or water, and contract gastrointestinal disease (Dandekar, Fieselmann et al. 2014). As a facultative anaerobe, it can survive in the different environment even with or without oxygen. There are millions cases of infection and thousand cases of fatal every year (Feasey, Dougan et al. 2012). Therefore, it is important to better understand how *Salmonella* adjust and adapt in different environments while attempt to colonization.

One of the adaptive strategy of *Salmonella* is using electron transport chain (ETC) as support bacteria to adapt and continue growth under toxicity condition (Husain, Bourret et al. 2008). This ETC contains cytochrome bo oxidase, cytochrome bd oxidase, NADH dehydrogenase and Succinate dehydrogenase (Fig. 1).

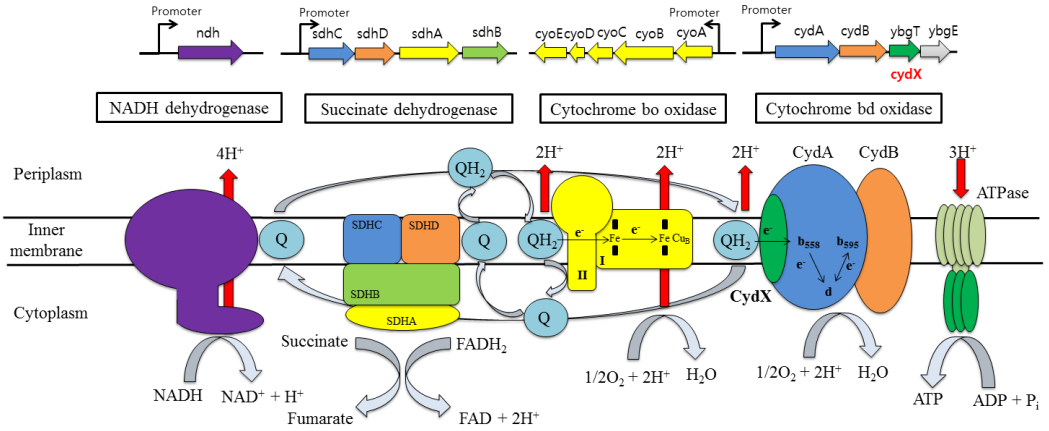


Figure 1. Electron transport chain in *Salmonella* including each component 's operon organization and functions

The cytochrome bd oxidase is an intergral membrane terminal oxidase of many prokaryotes, including several pathogens *Salmonella* Typhimurium, *Escherichia coli* (Junemann 1997). This bd oxidases utilize quinols as electron donors from the periplasmic side and protons from the intracellular side like heme-copper oxidases (HCOs), but do not pump protons (Borisov, Gennis et al. 2011). They have no copper, but carry three hemes: a low-spin heme b_{558}, two

high-spin hemes b_{595} and d (Borisov, Gennis et al. 2011). This enzyme complexes have a high oxygen affinity and thus helps bacteria continue aerobic respiration under low oxygen tension (Mason, Shepherd et al. 2009) (Fig. 2).

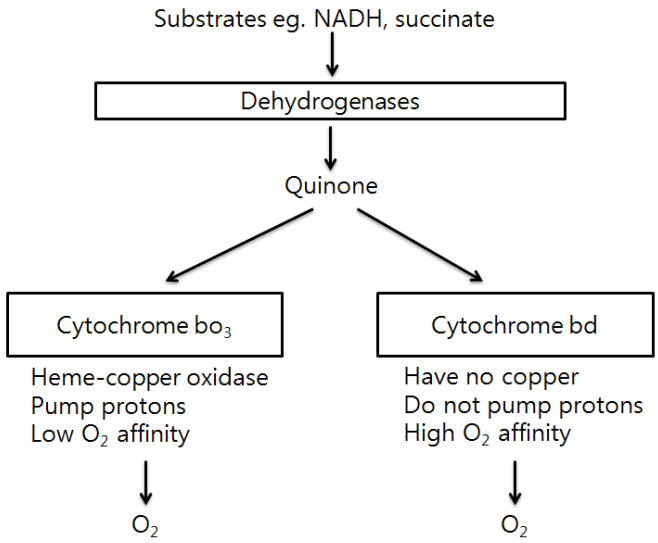


Figure 2. Comparison between cytochrome bo and cytochrome bd oxidase in their structure and properties

Cytchrome bd oxidases are made up by three subunits called CydA, CydB and until recently, ybgT renamed to CydX (Borisov, Gennis et al. 2011, Vanorsdel, Bhatt et al. 2013, Allen, Brenner et al. 2014) (Fig. 1). This small protein CydX plays a crucial role for the assembly and the stability of the active site di-heme center in *Escherichia coli, Brucella abortus,* and only for function but not assembly in *Shewanella oneidensis* (Sun, de Jong et al. 2012, Hoeser, Hong et al. 2014, Chen, Luo et al. 2015). Recently, the first structure of a bd-type oxidase complexed with cydX was described in *G. thermodenitrificans* (Chen, Luo et al. 2015, Safarian, Rajendran et al. 2016). Although the role of the small protein CydX have been reported as mentioned before but still not known in intracellular pathogen, *S.* Typhimurium. Therefore, the protein sequences of CydX were compared between different species and indicated that the protein sequences of CydX homologues in *Salmonella* is the most similar to this protein in *Escherichia coli* (Fig. 3). It suggest that the small protein CydX in *Salmonella* might also play a physiological role in cytochrome bd oxidase.

Escherichia coli	MWYFAWILGTLLACSFGVITALALEHVESGKTGQEDI
Salmonella Typhimurium	MWYFAWILGTLLACAFGIITALALEHVEAGKTGQEES
Shewanella oneidensis	MWYFTWILGVLLACAFGVINALWLENTENMDRSSDDPE
Brucella abortus	MWYFSWLLGLPLAAAFAVLNAMWYELMDDRARKRLA ADPTAELALEGNKHH
Geobacillus thermodenitrificans	MQTFLIMYAPMVVVALSVVAAFWVGLKDVHVNE

Figure 3. Alignment of protein sequences of CydX homologues from *Salmonella* and other bacteria species

Amino acids are colored based on their existence in protein sequences as follow: red amino acids appear in all species, green amino acid appear that homologues in comparison with *Escherichia coli.*

In *Escherichia coli*, the deletion of cydX led to slow bacterial growth in aerobic liquid culture, mixed-colony formation and increase sensitivity to ß-mercaptoethanol compared with cydAB mutant (Vanorsdel, Bhatt et al. 2013, Allen, Brenner et al. 2014). One of the main effects of ß-mercaptoethanol is reduction of disulfide bonds in periplasmic proteins. For maintaining the proper folding disulfide formation for periplasmic protein, many prokaryotes have evolved a well-regulated system, as well-known as Dsb pathway including DsbA and DsbB (Messens and Collet 2006) Both the cytochrome bo and cytochrome bd oxidase can support for this disulfide formation system under aerobic growth (Bader, Muse et al. 1999) (Fig. 4). There would be possibilities that (1) the CydAB pathway have a greater role in supporting Dsb-related oxidation or (2) the both complexes are required for wild-type levels of resistance to disulfide bond reduction (Vanorsdel, Bhatt et al. 2013).

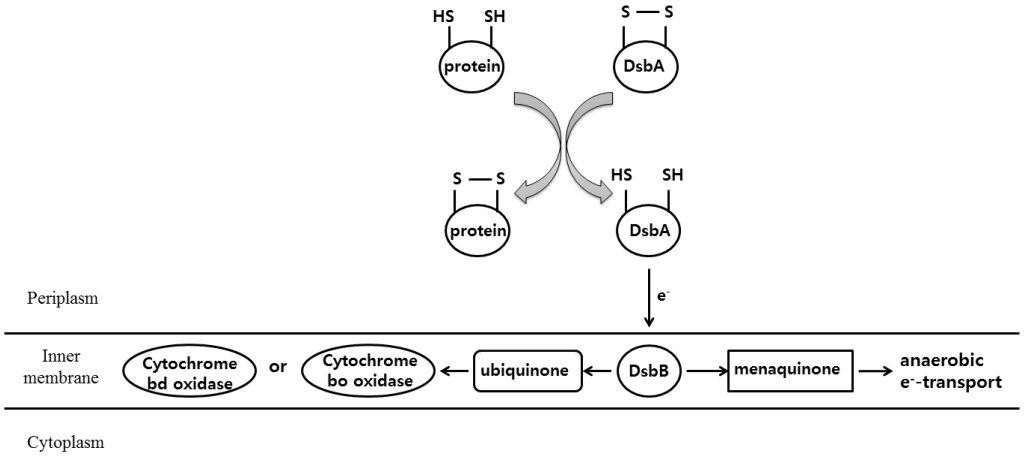


Figure 4. DsbB links disulfide bond formation to Electron transport chain

In this study, I have identified the role of the small protein cydX for the cytochrome bd oxidase in *Salmonella* Typhimurium and investigate the mechanism that cytochrome bd oxidase can protect bacteria from ß-mercaptoethanol, a reducing agent that disrupts disulfide bonds.

II. MATERIAL AND METHODS

1. Bacterial strains and culture media

Mutant strains used in this study are derivatives of *S.* Typhimurium 14028s and shown in Table 1. Strains were grown in Luria-Bertani (LB) (Difco, Detroit, MI, USA) medium at 37^0C with shaking.

For mutant construction and cloning, the following antibiotics were added to LB medium when appropriate: ampicillin (200 μg/ml), kanamycin (50 μg/ml) and chloramphenicol (20 μg/ml). *S.* Typhimurium mutant strains were constructed via the λ red method, described previously (Datsenko and Wanner 2000) with oligonucleotides listed in Table 2. All constructed mutation was confirmed by PCR analysis using gene-flanking primers then transduced via phage P22 into Wild-type 14028s. Complementation of the ΔcydABX, ΔcydX and ΔcydAB was achieved by cloning the cydABX, cydX and cydAB genes into pBAD33 and pBAD18, in respectively.

2. Plasmid construction

Complementation of the ΔcydABX, ΔcydX and ΔcydAB was achieved by cloning the cydABX, cydX and cydAB genes into pBAD33 and pBAD18, in respectively.

To make CydX overexpression plasmids, the short genes were amplified by PCR from *Salmonella* Typhimurium 14028s genomic DNA, and the cydX gene was then cloned into the pBAD33 plasmid. Positive transformants were screened by flanking primers, and plasmids with inserts were confirmed by sequencing.

Table 1. Bacterial strains and plasmids used in this study

Strains	**Relevant characteristic**	**Source or Reference**
IB1	*S.* Typhimurium strain 14028s Wild-type	ATCC
IB1006	14028s rpoS::CM	Lab collection
IB1370	14028s ΔcyoA::CM	Lab collection
IB1439	14028s ΔcydAB::FRT	Lab collection
IB1959	14028s ΔcydX::FRT	This study
IB1963	14028s ΔcydX::FRT/pBAD33-cydX	This study
IB1964	14028s ΔcydX::FRT/pBAD33	This study
IB1965	14028s ΔcydABX::KM	This study
IB1966	14028s ΔcydABX::FRT	This study
IB2024	14028sΔcydABX::KM/pBAD33-cydABX	This study
IB2032	14028s ΔcydAB::FRT/pBAD18-cydAB	This study
IB2033	14028s ΔcydAB::FRT/pBAD18	This study
IB2034	14028s ΔcydABX::KM/pBAD33	This study
IB2048	14028s ΔDsbA::KM ΔDsbB::CM	This study
Plasmid		
pKD3	Plasmid carrying the FRT-Cm^r-FRT-cassette	Datsenko and Wanner (2000)
pKD4	Plasmid carrying the FRT-Km^r-FRT-cassette	Datsenko and Wanner (2000)
pTP233	Plasmid encoding IPTG-inducible Lamda red recombinase	Poteete and Fenton (1984)
pcp20	Plasmid expressing the FLP recombinase	Cherepanov and Wackernagel (1995)
Host plasmid		

pBAD18(Amp^r)	cydAB open reading frame	This study
pBAD33(Cm^r)	cydABX open reading frame	This study
	cydX open reading frame	This study

Table 2. Oligonucleotides used in this study

Primer	**Sequence (5'-3')**
cydX-P1-FW	5'-ATGTGGTATTTCGCATGGATTCTGGGAAC GCTTCTTGCCTGTGTAGGCTGGAGCTGCTTC-3'
cydX-P2-Rev	5'-TTAGCTTTCTTCTTGACCGGTTTTACCCG CTTCAACGTGC CATATGAATATCCTCCTTAG-3'
cydX -Fw	5'-ATATTGAAAGCAACACCCAC-3'
cydX -Rev	5'-CATTACCGCATAAATATACT-3'
cydX -SalI-Rev	5'-TATTGCATGTCGACAATATGA-3'
cydAB-P1-Fw	5'- ACCACTTCCTGTTTGTGCCGCTAACGCTC GGTATGGCGTTGTGTAGGCTGGAGCTGCTTC-3'
cydAB-Fw	5'-ATTGTCCGTGATCAAATTCC-3'
cydAB-EcoRI-FW	5'-GCCTTATCGAATTCGTCTAT-3'
DsbA-P1-FW	5'-ATGAAAAAGATTTGGCTGGCGCTGGCTGGT ATGGTTTTAG GTGTAGGCTGGAGCTGCTTC-3'
DsbA-P2-Rev	5'-ACATCCATGCTGCTCGTATCCATGCCTTGT GGGTTAATCT CATATGAATATCCTCCTTAG-3'
DsbA-Fw	5'-CGACAGACAACGACTTTTAT-3'
DsbA-Rev	5'- CAACGTAAGCTTAATACGCT-3'
DsbB-P1-FW	5'-CAGCACGTCATGTTACTTAAACCTTGCGT GCTATGTATTTGTGTAGGCTGGAGCTGCTTC-3'
DsbB-P2-Rev	5'-CGACCGAACAGGTCGCGTTTTTTAGGCTT AAATGCCTGGGCATATGAATATCCTCCTTAG-3'
DsbB-Fw	5'- GCCGGCCACCGAATGGATGA-3'
DsbB-Rev	5'- ACGGCAAACAAAGCTTCGG-3'
pBAD-FW	5'- TCGCAACTCTCTACTGTTTC-3'
pBAD-Rev	5'- AGACCGCTTCTGCGTTCTGA-3'

3. Measurement of the susceptibility to ß-mercaptoethanol

The effects of the ß-mercaptoethanol on growth of wild type and *S.* Typhimurium mutants were recorded spectrometrically on a Bioscreen C microbiology Microtiter plate Reader. *Salmonella* cultures grown overnight were normalized to 0.02 $O.D_{600}$ by phosphate-buffered saline (PBS) then grown in LB broth treated with or without 10 mM ß-mercaptoethanol. The half-life ($t_{1/2}$) of ß-mercaptoethanol at pH 6.5 is more than 100 h. Bacterial growth was monitored by assaying the $O.D_{600nm}$ every 30 min for 24 hours at 37^{o}C in shaking incubation.

4. Oxygen consumption

Strains grown overnight in LB broth were diluted 1:200 in LB broth and grown at 37^{o}C in a shaker incubator until their OD_{600} reached to 0.5. These cultures were then transferred into an air-sealed, multiport measurement chamber equipped with an Iso-Oxy-2 oxygen sensor. The variation of O_2 in the cultures was monitored with a TBR4100 free radical analyzer (World Precision Instruments, Inc., Sarasota, FL, USA). To determine the effect of ß-mercaptoethanol on bacterial respiration, O_2 consumption was observed in cultures treated for 1 min or since starting culture with 10 mM ß-mercaptoethanol. The data are expressed as micromolar concentration of O_2.

5. Cytochrome spectrometry

WT and mutant *Salmonella* strains lacking the cytochrome bo oxidase ($bo^{-}bd^{+}$) or lacking the cytochrome bd oxidase ($bo^{+}bd^{-}$) was grown overnight in LB broth then sub-cultured in LB broth until reach $O.D_{600}$ of 0.5. Membranes preparations from these mutants were prepared as described previously (Husain, Bourret et al. 2008). Briefly, bacterial pellets were re-suspended in 10 mM EDTA, 100 mM Tris-HCl Buffer, pH 8.5. Cell debris was discarded by centrifuging for 30 min at 27,000x g in a Sorvall SL-50T rotor. The supernatant was centrifuged at 200,000 x g in a Beckman-type MLA-130 rotor for 1 h and the pellet solubilized in 75 mM potassium phosphate, 150 mM KCl, 5 mM EDTA, and 60 mM N-dodecyl-N,N-dimethyl-3-ammonio-1-

propane sulfonate (Sigma-Aldrich, St.Louis, Missouri, USA) buffer, pH 6.4. The solution was centrifuged at 200,000x g for 1 h. Supernatants containing inner membranes were collected, and the protein concentrations were determined using a Bradford protein assay (Bio-rad, Hercules, California, USA). The protein was adjusted to 1.5 mg/ml in 75 mM potassium phosphate, 150 mM KCl, 5 mM EDTA, 10 mM ascorbate, and 60 mM N-dodecyl-N,N-dimethyl-3-ammonio-1-propane sulfonate buffer, pH 6.4. Absorbance spectroscopy was collected in a Hitachi U-1900 spectrophotometer and analyzed using UV solutions software. Selected groups of proteins were treated with 5 mM ß-mercaptoethanol for 10 min before the absorption spectra were collected. The air-oxidized sample was used as a negative control.

6. TMPD oxidation assay

Oxidase assay were performed as described previously (Vanorsdel, Bhatt et al. 2013). In briefly, prepared membrane fraction were added to a fresh solution containing 1% N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) and 0.16 mM ascorbate. The protein content in each sample of an individual assay was normalized and then each sample was tested in quadruplicate reaction. Blank tubes has only buffer to monitor oxidation of the TMPD by air. Oxidation of TMPD activity was measured spectrophotometrically at absorbance of 611 nm on an Epoch Microplate reader (BioTek Instruments, Winooski, VT, USA).

III. RESULTS

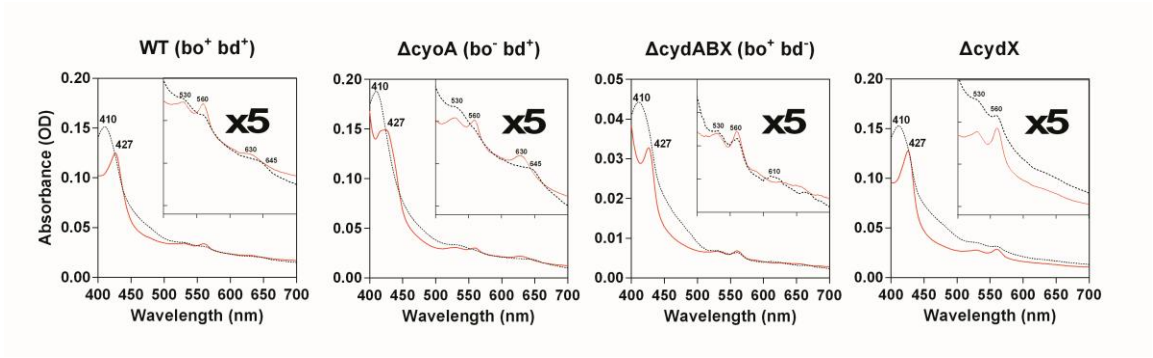
1. CydX is required for cytochrome bd oxidase activity and assembly in *S.* Typhimurium

To confirm the role of small protein CydX in cytochrome bd oxidase, there are possibility that this small protein can be required for cytochrome bd oxidase ‘s assembly or function or both. In order to test the cydX requirement for this enzyme complex ‘s assembly, the spectral properties was measured from the membrane fraction purified from WT, ΔcyoA (bo^-bd^+), ΔcydABX (bo^+bd^-) and ΔcydX mutant. Under air-oxidized form, the WT and ΔcyoA mutant presented a Soret maximum at 410nm and a distinct shoulder near 645 nm, while in the reduced form with sodium dithionite these peaks altered to 427, 530, 560 nm and 630 nm. In other hand, in both ΔcydABX and ΔcydX mutants, the spectrum of oxidized form had Soret maximum at 410 nm and another peak near 610 nm, and in that of the reduced form, the peaks were shifted to 427, 531, and 560 nm, with increase in their absorption intensities (Fig. 5). This result shown that WT and ΔcyoA mutant have typical spectrum of full cytochrome bd oxidase, while ΔcydABX mutant had typical spectrum of full cytochrome bo oxidase. These spectral properties were consistent with previous findings in *Escherichia coli* (Kita, Konishi et al. 1984, Forte, Borisov et al. 2017). This is the first evidence of spectral properties of both cytochrome bo and bd oxidase in *Salmonella* Typhimurium. The ΔcydX mutant shown the spectral properties similar with ΔcydABX mutant in the loss heme d in peak of 645 nm, it suggested that cydX maybe required for cytochrome bd oxidases’ assembly and/or the stability.

To investigate the role of small protein CydX in cytochrome bd oxidase activity, a ΔcydX mutant in *S.* Typhimurium was tested the cytochrome bd oxidase phenotypes. The loss of CydX caused the mixed-colony formation and reduced the cytochrome bd-I oxidase acitivity to a half level of WT (Figs. 6A and 6B). And then this mutant was treated with ß-mercaptoethanol, hydroxylamine, hydrogen peroxide, spermine NONOate and its sensitivity

was compared in ΔcydAB and ΔcydABX mutants. Under low oxygen condition (up to 12% oxygen level), the addition of 10 mM ß-mercaptoethanol to the media delayed the growth of these 3 mutants by 3 folds in comparison with the wild type (Fig. 6C). Based on the spermine NONOate (sNO) growth data, these mutants also displayed the sensitivity in presence of 2 mM sNO (Fig. 6C). However, none of them showed significant sensitivity to hydroxylamine, hydrogen peroxide (data not shown). Moreover, the deletion of cydABX, cydAB or cydX genes enhanced the bacterial tolerance to kanamycin, an aminoglycoside antibiotic drug, compared with wild type (Fig. 6D). The mutants lacking whole or a part of cytochrome bd oxidase caused the modification in bacterial membrane, then led to alter the proton motivate force and decrease the kanamycin uptake. Additionally, overexpression of the cydX protein in ΔcydX mutant was able to restore the resistance to ß-mercaptoethanol (Fig. 7). Together, these data suggest that the CydX protein is required for cytochrome bd oxidase activity and maybe for assembly.

(A)



(B)

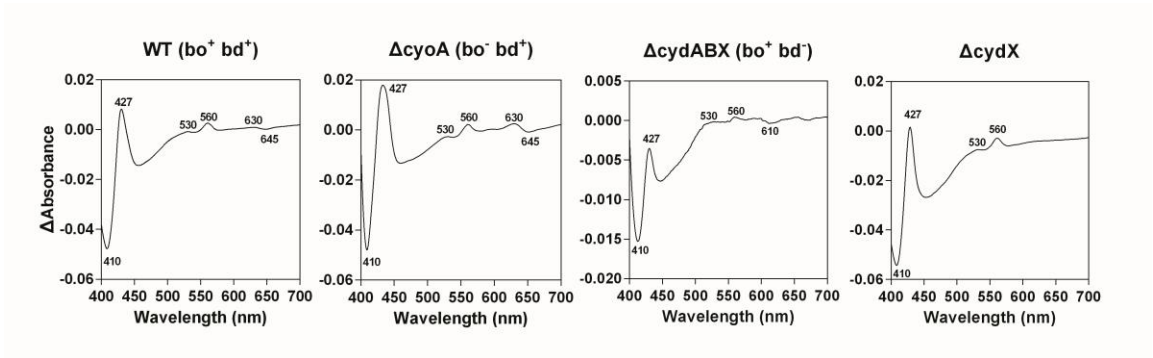
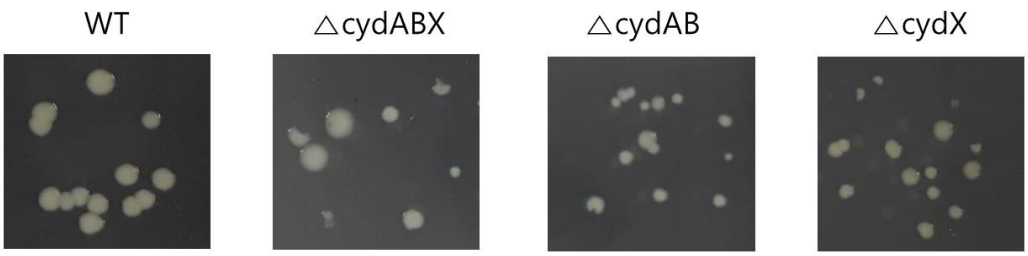


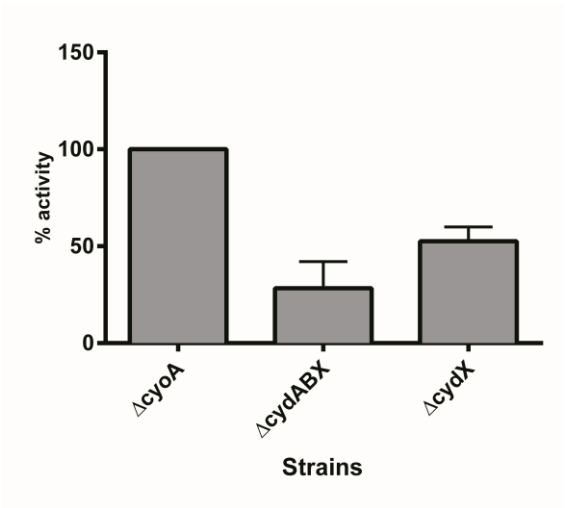
Figure 5. UV-VIS spectroscopy of membrane fraction purified from *S.* Typhimurium.

(A) Absolute spectra of WT ($bo^{+}bd^{+}$), ΔcyoA($bo^{-}bd^{+}$), ΔcydABX ($bo^{+}bd^{-}$), and ΔcydX strains at room temperature. The absorption spectra of the oxidized in air (black dot), and reduced with excess of sodium dithionite (red line) in the region between 400nm and 700nm. (B) UV-VIS difference spectrum of the reduced-minus-oxidized.

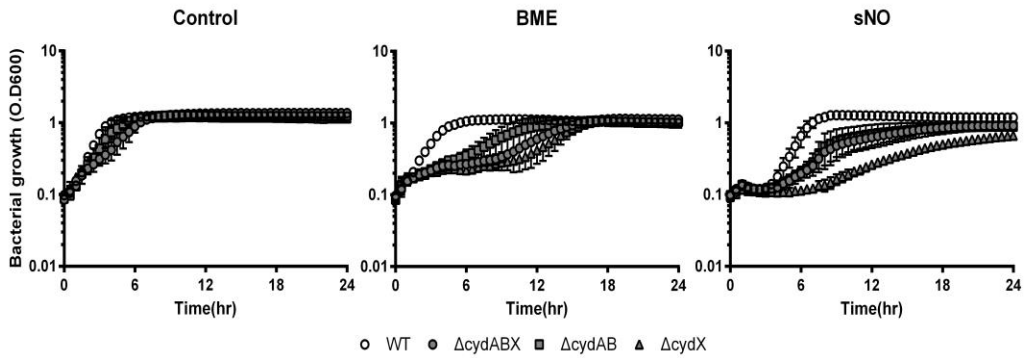
(A)



(B)



(C)



(D)

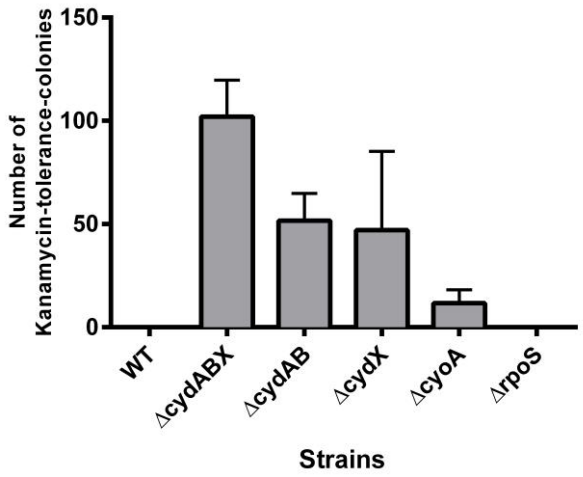


Figure 6. The small protein of CydX is crucial for cytochrome bd oxidase activity.

(A) Mixed-colony formation in ΔcydABX, ΔcydAB, and ΔcydX mutant strains in LB plates grown at 37^{o}C overnight. (B) Cytochrome bd oxidase activity. Cell membrane fractions were prepared as described in Material and methods. The oxidase activity was assayed using the increase of absorbance at 611 nm subtracted with blank. (C) ΔcydX mutant strains are hypersusceptible to BME and spermine NONOate (sNO). WT and mutant strains were grown in LB media in the presence or absence of 10 mM BME or 2 mM sNO. Data shown are the mean O.D. ± standard deviation (SD) from three independent experiments. UT, untreated. (D) The number of kanamycin resistant colonies grown overnight.

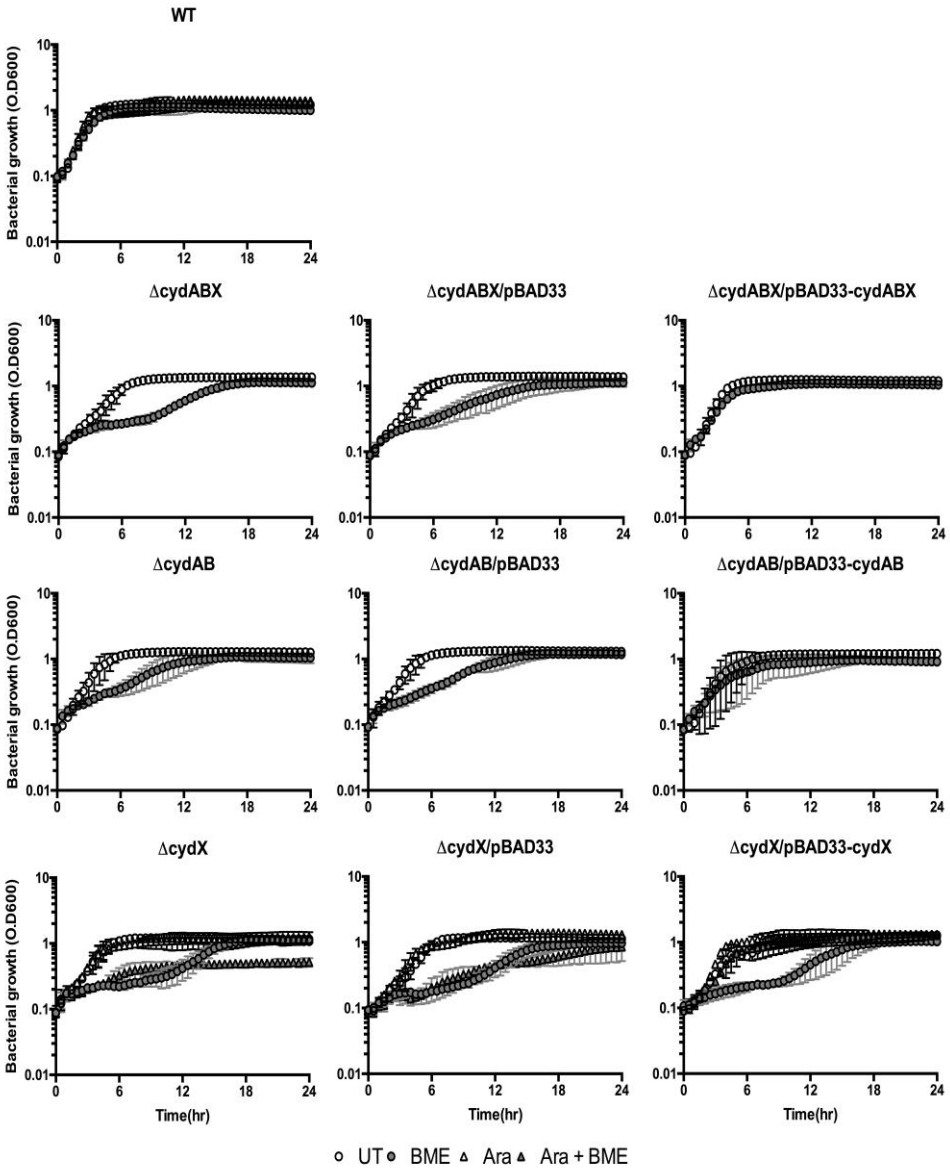


Figure 7. Complementation of cydX mutation.

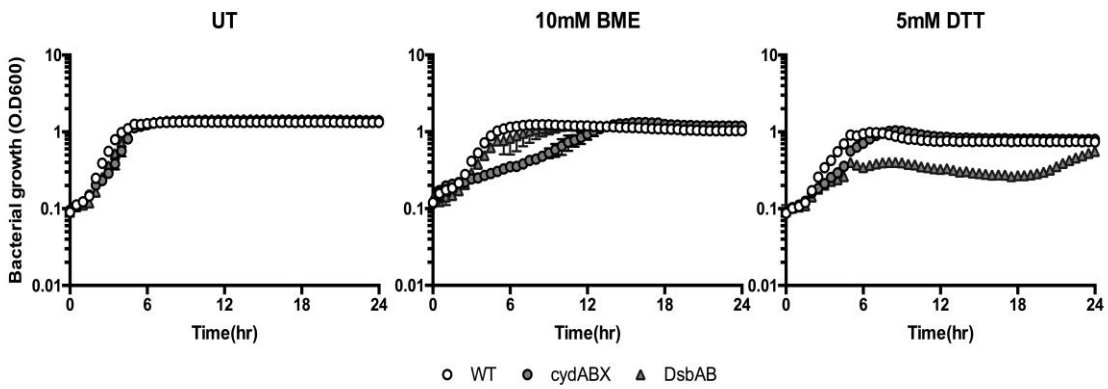
CydX mutants and those harbouring cydX clone plasmid were grown in LB broth supplement with or without 0.2% arabinose, which can induce P_{Ara} promoter. Data shown are the average of time to half-maximal O.D600nm ± standard deviation (SD) from three independent experiments.

2. CydABX mutants Salmonella is not susceptible to other reductant DTT

To confirm whether these mutants are sensitive to ß-mercaptoethanol related with reducing activity or not, the ΔcydABX mutant was tested with DTT (redithiothreitol), another redox reagent-reducing sulfide. A ΔDsbA ΔDsbB double mutant was constructed as DTT-sensitive positive mutant. Contrary to expectation, 5 mM DTT addition virtually stopped the growth of ΔDsbA ΔDsbB but not ΔcydABX (Fig. 8A). It proposed that the susceptibility of ΔcydABX mutant might not relate with reducing activity of ß-mercaptoethanol.

Glucose and glycerol are known as the main carbon source for the bacterial biosynthesis pathway. While glycerol enhance bacterial respiration by facilitating quinone redirection in electron transport chain (van Beilen and Hellingwerf 2016), glucose fermentation can promote bacterial growth when the respiration is impaired. Supplement with glucose, but not glycerol, can enhance the growth of ΔcydABX mutant in the presence of ß-mercaptoethanol (Fig. 8B)..This result suggested that ß-mercaptoethanol might directly inhibit bacterial respiration but not through the disulfide bond breakage.

(A)



(B)

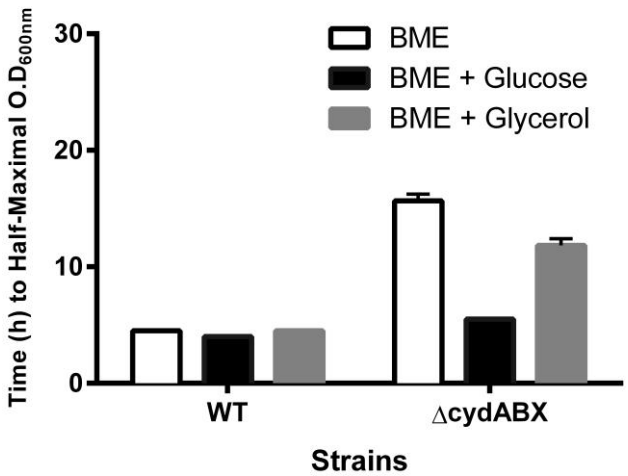


Figure 8. The differential effect of reducing agents on the growth of ΔcydABX mutant.

(A) Susceptibility of ΔcydABX mutant to ß-mercaptoethanol and DTT. WT, ΔcydABX, ΔDsbAB were grown in LB treat with 10 mM BME or 5 mM DTT. Data represent the mean O.D. ± standard deviation (SD) of 4 observations from two independent cultures. (B) Glucose fermentation can recover bacterial growth when respiration is impeded by ß-mercaptoethanol. WT and ΔcydABX mutant ‘s growth curve was monitored in medium containing LB broth supplemented with 50 mM MES adjusted to pH 7.0 before adding 0.4% Glucose or 0.4% Glycerol in the presence of 20 mM ß-mercaptoethanol. Data are the mean time to ½ maximal O.D. 600nm of 6 observations from two independent experiments. Error bar represent standard error.

3. Inhibition effect of ß-mercaptoethanol to bacterial respiration

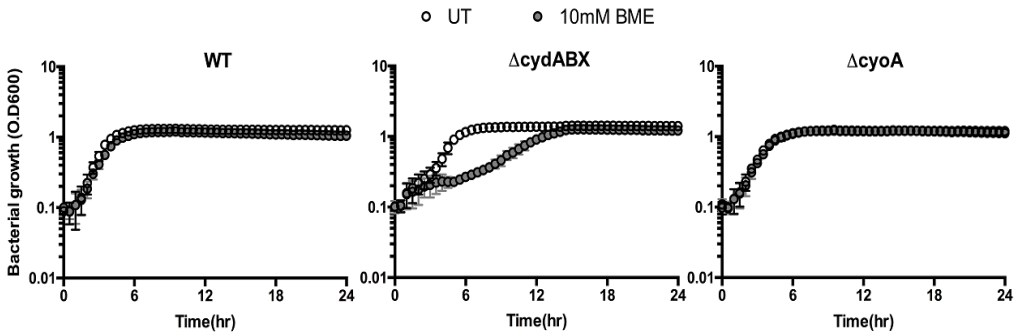
Cytochrome bo oxidase is a heme-copper oxidase (HCO) with a four subunit transmembrane complex that oxidizes ubiquinone and utilizes electrons to reduce oxygen to water (Blanca, Jon et al. 1994). This enzyme complex binds molecular oxygen between its Cu_B copper atom and heme o_3 cofactor. Unlike HCOs, cytochrome bd oxidase is bacterial evolution that can support bacterial continue growth under hypoxic conditions. But the impact of ß-mercaptoethanol on cytochrome bo or bd oxidase in bacteria has not reported. Both ΔcyoA mutant (lacking cytochrome bo oxidase) and ΔcydABX mutant (lacking cytochrome bd oxidase) grew similar in LB broth under low oxygen condition, indicating that either one of the two cytochrome oxidase is sufficient for bacterial respiration. The addition of 10 mM ß-mercaptoethanol caused growth impairment in ΔcydABX mutant but not in ΔcyoA mutant (Fig. 9A). This result suggests that in the presence of ß-mercaptoethanol, cytochrome bo oxidase might lose activity, but cytochrome bd oxidase does not.

I next examined the effect of ß-mercaptoethanol to respiration of ΔcyoA, ΔcydABX and ΔcydX mutants. Oxygen consumption of these mutants were monitored with a chamber equipped with an Iso-Oxy-2 O_2 probe. As predicted, ß-mercaptoethanol addition inhibited respiration in ΔcydABX mutant lacking cytochrome bd oxidase that depended upon cytochrome bo oxidase for oxidase activity (Fig. 9B).

There is a possibility that heme o_3 with Cu_B copper atom of cytochrome bo oxidase might be targeted by ß-mercaptoethanol. Spectroscopy of membrane fraction purified from ΔcydABX ($bo^+ bd^-$) showed a loss in the absorption band of 610 nm displaying the loss of heme o_3 upon ß-mercaptoethanol treatment (Fig. 10A). This results indicates that ß-mercaptoethanol targets cytochrome bo oxidase, therefore, inhibits bacterial respiration in the absence of cytochrome bd oxidase.

As previous finding, 6 mM exogenous copper is toxic to *Escherichia coli* (Steck and Steck 2001) and is able to react with ß-mercaptoethanol to form the precipitation of copper mercaptide (Sarma and Nageswar 1981). Thus, the 1mM $CuCl_2$ was used to not cause toxic to bacteria as well as form precipation with ß-mercaptoethanol . The growth of ΔcydABX mutant was largely recovered by supplementing $CuCl_2$ into the culture containing ß-mercaptoethanol (Fig. 10B).

(A)



(B)

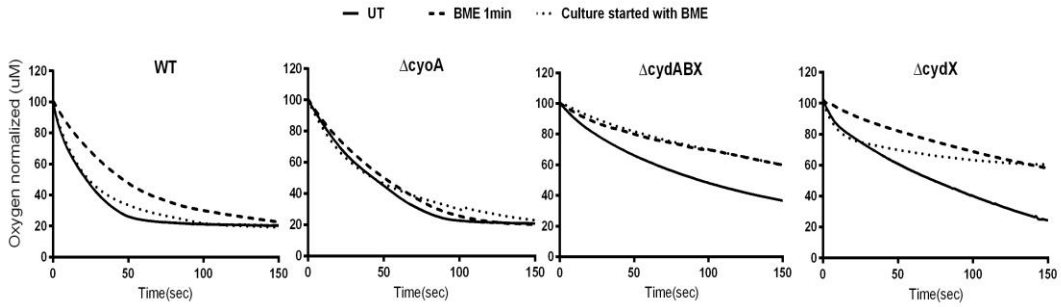
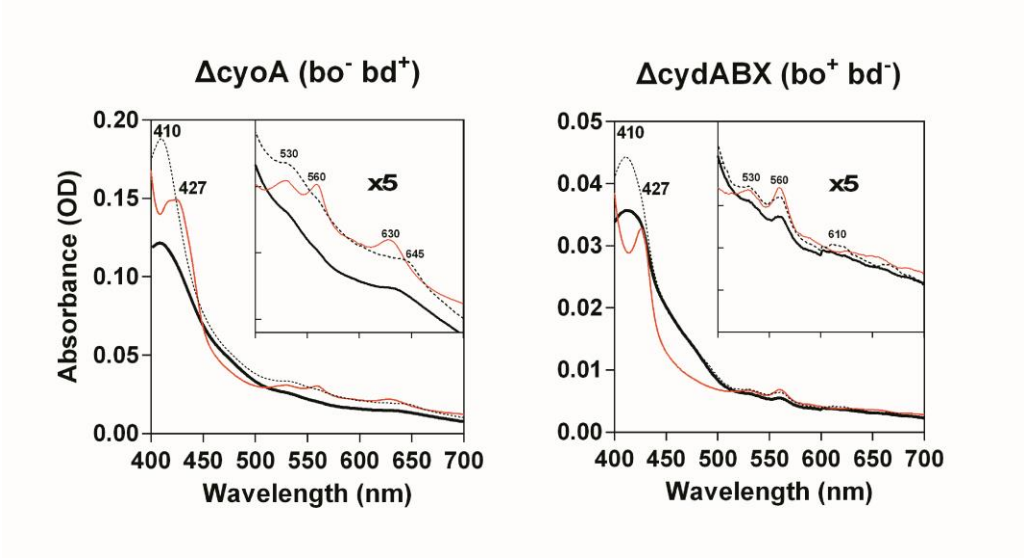


Figure 9. ß-mercaptoethanol inhibits cytochrome bo oxidase activity but not cytochrome bd oxidase.

(A) WT (bo^+ bd^+), ΔcydABX (bo^+ bd^-), Δcyo (bo^- bd^+) were grown in LB treated with 10 mM BME. Data are the representative of 10 observations from 5 independent cultures. (B) Bacterial cultures were grown in LB broth until OD600 of 0.5 and O2 consumption were monitored over time. Prior to analysis, bacteria were treated with 10 mM BME for 1 min or treated since started culture. Control, BME 1 min-treated and BME started-culture treated are shown for comparison. Data are representative of three independent experiments.

(A)



(B)

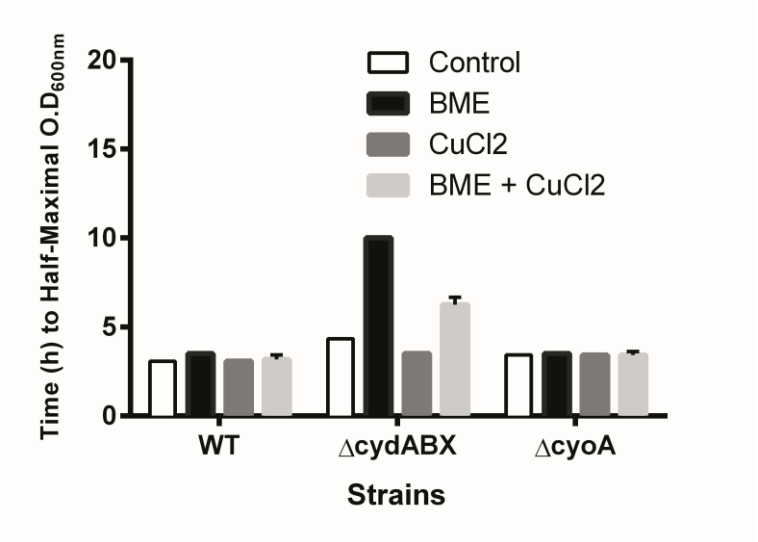


Figure 10. ß-mercaptoethanol impairs cytochrome bo oxidase.

(A) The absorption spectra of purified membrane air-oxidized (black dot), reduced with excess sodium dithionite (red line), treated with 5 mM BME (black bold line) from early stationary phase ΔcyoA (bo- bd+) and ΔcydABX (bo+ bd-) *Salmonella* mutant strains grown in LB broth. Membrane preparation was treated with 5 mM ß-mercaptoethanol for 10 min before spectrophotometric analysis. This magnified inset shows a detail of the 500 to 700 nm region. (B) Exogenous copper (1 mM $CuCl_2$) partly eliminates the lag phase caused by 10 mM BME in ΔcydABX mutant.

IV. DISCUSSION AND CONCLUSION

In this study, we have shown that the small protein CydX is required for cytochrome bd oxidase function, particularly in resistance to ß-mercaptoethanol, and may be required for cytochrome bd oxidase assembly. This enzyme complex plays a great role in protecting bacterial respiration to against this reductant, while cytochrome bo oxidase containing Cu_B copper atom would not.

There are two cytochrome bd oxidase complexes: the cytochrome bd-I oxidase complex encoded by the cydA-cydB-ybgT-ybgE (cydABTE) operon (Fig. 1) and the cytochrome bd-II oxidase complex encoded by the appC-appB-yccB-appA (appCBBA) operon reported in *Escherichia coli*. The expression of cydX was induced ~ 4- to 8-fold while ~ 30-fold for appX during growth under low oxygen condition compare to aerobic condition (Hemm, Paul et al. 2010). Moreover, it was indicated that overexpression of appX compensates the cydX deletion in *E. coli* (Vanorsdel, Bhatt et al. 2013). However, under low oxygen condition, the loss of cydX still led to sensitivity to ß-mercaptoethanol in *Salmonella* (Fig. 6B). Although the existence of cytochrome bd-II in *Salmonella* was still unknown, it is likely that the cytochrome bd-II in *Salmonella* might not play any physiologically role in resistance to ß-mercaptoethanol.

Cytochrome bo oxidases have structure similar to aa_3 type family of cytochrome c oxidases in mammals (Saraste, Raitio et al. 1988, Chepuri, Lemieux et al. 1990). Cytochrome bo complex‘s function can be damaged by many ligands. It has been noted that this oxidase was modestly resistant to nitric oxide and cyanide, which are competitive inhibitors of oxygen binding (Mason, Shepherd et al. 2009). Hydrogen sulfide binds directly to the ferric (Fe^{III}) heme a_3 center and completely inhibits cytochrome c oxidase activity (Nicholls 1975, Nicholls and Kim 1982, Hill, Woon et al. 1984). Imidazole and b-mercaptoethanol can bind to cytochrome c_2 from *Rhodobacter capsulatus* (Dumortier, Fitch et al. 2002). Additionally, it is reported that other mercaptans can form transient complexes with cytochrome c at pH 7.2 or

even at low pH (Jafar and Shimizu 1987, Tomková, Antalík et al. 1992). In our study, the spectrum of cytochrome bo membrane fraction have shown that the high-spin heme o_3 with copper Cu_B might be targeted by ß-mercaptoethanol while this reagent ruined the peak 610nm (Fig. 10).

Cytochrome bd oxidase support to resist in nitrosative stress and synergic with hmp to *Salmonella* pathogensis (Jones-carson, Husain et al. 2016). The deletion of a bd oxidase in *Mycobacterium tuberculosis* led to hypersensitivity to bedaquiline, a tuberculosis drug that inhibits the F_1F_o-ATP synthase, highlights the potential of bd oxidases as drug targets. Furthermore, they also pointed out that Aurachin D can inhibit cytochrome bd activity at sub-micromolar concentrations, suggested the feasibility of this important new drug target (Lu, Heineke et al. 2015). In contrast, my study showed that deletion of cytochrome bd oxidase in *Salmonella* led to the increasing tolerane to kanamycin (Fig. 6D). It might be caused by interrupting the proton motive force that is required for drug uptake. This evidence is consistent with the same observation that NO-mediated repression of respiratory activity induces bacterial resistances to aminolglycosides (McCollister, Hoffman et al. 2011). Therefore, the using drug targeted to cytochrome bd oxidase should be considered carefully when used combination with aminoglycosides antibiotic drug.

In conclusion, my investigations indicate the requirement of the small protein CydX in cytochrome bd oxidase function and assembly. This enzyme complex play an important role as a key survival factor for bacteria under various toxic conditions. Thus, the cytochrome bd oxidase will be a potential drug target for antimicrobial therapy in near future in order to against the wide spreading of antibiotic resistance bacteria among the world.

V. REFERENCES

Allen, R. J., E. P. Brenner, C. E. VanOrsdel, J. J. Hobson, D. J. Hearn and M. R. Hemm (2014). "Conservation analysis of the CydX protein yields insights into small protein identification and evolution." BMC genomics **15**(1): 946-946.

Bader, M., W. Muse, D. P. Ballou, C. Gassner and J. C. A. Bardwell (1999). "Oxidative protein folding is driven by the electron transport system." Cell **98**(2): 217-227.

Blanca, J. A. G.-h., B. Jon, J. Ma and R. B. Gennis (1994). "MINIREVIEW The Superfamily of Heme-Copper Respiratory Oxidases." **176**(18): 5587-5600.

Borisov, V. B., R. B. Gennis, J. Hemp and M. I. Verkhovsky (2011). "Biochimica et Biophysica Acta The cytochrome bd respiratory oxygen reductases." BBA - Bioenergetics **1807**(11): 1398-1413.

Chen, H., Q. Luo, J. Yin, T. Gao and H. Gao (2015). "Evidence for the requirement of CydX in function but not assembly of the cytochrome bd oxidase in Shewanella oneidensis." Biochimica et Biophysica Acta - General Subjects **1850**(2): 318-328.

Chepuri, V., L. Lemieux, D. C. T. Au and R. B. Gennis (1990). "The Sequence of the Cyo Operon Indicates Substantial Structural Similarities between the Cytochrome-O Ubiquinol Oxidase of Escherichia-Coli and the Aa3-Type Family of Cytochrome-C Oxidases." Journal of Biological Chemistry **265**(19): 11185-11192.

Cherepanov, P. P. and W. Wackernagel (1995). "Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant." Gene **158**(1): 9-14.

Dandekar, T., A. Fieselmann, E. Fischer, J. Popp, M. Hensel and J. Noster (2014). "Salmonella-how a metabolic generalist adopts an intracellular lifestyle during infection." Frontiers in cellular and infection microbiology **4**(January): 191-191.

Datsenko, K. A. and B. L. Wanner (2000). "One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products." Proceedings of the National Academy of Sciences of the United States of America **97**(12): 6640-6645.

Dumortier, C., J. Fitch, T. E. Meyer and M. A. Cusanovich (2002). "Protein dynamics: Imidazole and 2-mercaptoethanol binding to the Rhodobacter capsulatus cytochrome c2 mutant, glycine 95 proline." Archives of Biochemistry and Biophysics **405**(2): 154-162.

Feasey, N. A., G. Dougan, R. A. Kingsley, R. S. Heyderman and M. A. Gordon (2012). "Invasive non-typhoidal salmonella disease: An emerging and neglected tropical disease in Africa." The Lancet **379**(9835): 2489-2499.

Forte, E., V. B. Borisov, J. B. Vicente and A. Giuffrè (2017). "Cytochrome bd and Gaseous Ligands in Bacterial Physiology." Advances in Microbial Physiology **71**: 171-234.

Hemm, M. R., B. J. Paul, J. Miranda-rı, A. Zhang, N. Soltanzad and G. Storz (2010). "Small Stress Response Proteins in Escherichia coli : Proteins Missed by Classical Proteomic Studies □ †." **192**(1): 46-58.

Hill, B. C., T. C. Woon, P. Nicholls, J. Peterson, C. Greenwood and A. J. Thomson (1984). "Interactions of sulphide and other ligands with cytochrome c oxidase. An electron-paramagnetic-resonance study." The Biochemical journal **224**(2): 591-600.

Hoeser, J., S. Hong, G. Gehmann, R. B. Gennis and T. Friedrich (2014). "Subunit CydX of Escherichia coli cytochrome bd ubiquinol oxidase is essential for assembly and stability of the di-heme active site." FEBS Letters **588**(9): 1537-1541.

Husain, M., T. J. Bourret, B. D. McCollister, J. Jones-Carson, J. Laughlin and A. Vázquez-Torres (2008). "Nitric oxide evokes an adaptive response to oxidative stress by arresting respiration." Journal of Biological Chemistry **283**(12): 7682-7689.

Jafar, A. B. U. and T. Shimizu (1987). "Thiol-coordinated Cytochrome Heme Octapeptides of Cytochrome c ; Model Compounds of." **135**: 109-113.

Jones-carson, J., M. Husain, L. Liu, D. J. Orlicky and A. Vázquez-torres (2016). "-Dependent Bioenergetics and Antinitrosative Defenses in." **7**(6): 1-10.

Junemann, S. (1997). "Cytochrome bd terminal oxidase."

Kita, K., K. Konishi and Y. Anraku (1984). "Terminal Oxidases of Escherichia coli Aerobic Respiratory Chain." J. Biol. Chem. **259**(5): 3368-3374.

Lu, P., M. H. Heineke, A. Koul, K. Andries, G. M. Cook, H. Lill, R. van Spanning and D. Bald (2015). "The cytochrome bd-type quinol oxidase is important for survival of Mycobacterium smegmatis under peroxide and antibiotic-induced stress." Scientific reports **5**(April): 10333-10333.

Mason, M. G., M. Shepherd, P. Nicholls, P. S. Dobbin, K. S. Dodsworth, R. K. Poole and C. E. Cooper (2009). "Cytochrome bd confers nitric oxide resistance to Escherichia coli." Nat Chem Biol **5**(2): 94-96.

McCollister, B. D., M. Hoffman, M. Husain and A. Vázquez-Torres (2011). "Nitric oxide protects bacteria from aminoglycosides by blocking the energy-dependent phases of drug uptake." Antimicrobial Agents and Chemotherapy **55**(5): 2189-2196.

Messens, J. and J.-F. Collet (2006). "Pathways of disulfide bond formation in Escherichia coli." Ijbcb **38**: 1050-1062.

Nicholls, P. (1975). "Inhibition of Cytochrome c Oxidase by Sulphide." Biochem. Soc. Trans. **3**: 316-319.

Nicholls, P. and J. K. Kim (1982). "Sulphide as an inhibitor and electron donor for the cytochrome c oxidase system." Canadian journal of biochemistry **60**(6): 613-623.

Poteete, A. R. and A. C. Fenton (1984). "Lambda red-dependent growth and recombination of phage P22." Virology **134**(1): 161-167.

Safarian, S., C. Rajendran, H. Müller, J. Preu, J. D. Langer, S. Ovchinnikov, T. Hirose, T. Kusumoto, J. Sakamoto and H. Michel (2016). "Structure of a bd oxidase indicates similar mechanisms for membrane- integrated oxygen reductases." Science **352**(6285): 583-586.

Saraste, M., M. Raitio, T. Jalli, V. Chepuri, L. Lemieux and R. B. Gennis (1988). "Cytochrome O from Escherichia coli Is Structurally Related to Cytochrome aa3." Annals of the New York Academy of Sciences **550**(1): 314-324.

Sarma, R. L. and S. Nageswar (1981). "1 . I n t r o d u c t i o n 2 . E x p e r i m e n t a l details." **12**: 377-382.

Steck, T. R. and T. R. Steck (2001). "Concentrations of Copper Thought To Be Toxic to." Society **67**(11): 5325-5327.

Sun, Y.-H., M. F. de Jong, A. B. den Hartigh, C. M. Roux, H. G. Rolán and R. M. Tsolis (2012). "The small protein CydX is required for function of cytochrome bd oxidase in Brucella abortus." Frontiers in cellular and infection microbiology **2**(April): 47-47.

Tomková, A., M. Antalík, J. Bágel'ová, P. Miskovský and J. Ulicný (1992). "Absorption and Raman spectroscopy study of cyt c-thiol complexes in acidic solutions." General Physiology and Biophysics **11**(3): 273-286.

van Beilen, J. W. A. and K. J. Hellingwerf (2016). "All three endogenous quinone species of Escherichia coli are involved in controlling the activity of the aerobic/anaerobic response regulator ArcA." Frontiers in Microbiology **7**(SEP): 1-11.

Vanorsdel, C. E., S. Bhatt, R. J. Allen, E. P. Brenner, J. J. Hobson, A. Jamil, B. M. Haynes, A. M. Genson and M. R. Hemma (2013). "The escherichia coli CydX protein is a member of the CydAB cytochrome bd oxidase complex and is required for cytochrome bd oxidase activity." Journal of Bacteriology **195**(16): 3640-3650.

VI. 국문초록

살모넬라의 cytochrome bd oxidase 활성에 필수적인 작은 단백질 CydX 의 역할

Kieu Minh Duc

지도교수 : 방 일 수

조선대학교 대학원

치의생명공학과

Cytochrome bd oxidase 는 저산소 조건에서 원핵 생물의 생존에 필요한 말단 산화 효소 복합체이다. 이 효소 복합체는 CydA, CydB 및 CydX 로 이름이 바뀐 작은 단백질 ybgT 를 포함하여 3 개의 서브 유닛을 갖는다. 이 작은 단백질 CydX 의 역할은 Escherichia coli 및 다른 종에서 보고되었지만 원핵 병원체인 Salmonella Typhimurium 에서는 아직 명확하게 알려지지 않았다. 이번 연구에서 우리는 이전 발견과 같이 CydX 가 효소 복합체의 일부이며 Escherichia coli 에서와 같이 *S.* Typhimuriu 에서 산화 효소 활성과 조립에 필요하다는 것을 확인했다. 또한, 환원된 Cytochrome bd oxidase 는 호기성 및 저산소 조건 모두에서 ß-mercaptoethanol (환원제)에 민감하다는 것을 확인했다. 우리는 ß-mercaptoethanol 의 민감성이 세균 호흡의 억제에 기인하고 disulfide bond 파괴에

기인하지 않는다는 것을 제안한다. cytochrome bd oxidase 는 박테리아의 호흡 활동을 보호하고 β-mercaptoethanol 은 cytochrome bo oxidase 의 heme-copper 를 표적으로 삼는다. 이는 cytochrome bd oxidase 가 가까운 장래에 항생제 치료를 위한 잠재적 약물 목표가 될 수 있다고 제안했다.