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ABSTRACT

EFFECT OF NUTRITION AND GROWTH CONDITIONS
ON D-CYCLOSERINE SENSITIVITY OF *ESCHERICHIA COLI*

Poornima Vasireddy, M.S.
Department of Biological Sciences
Northern Illinois University, 2017
Dr. R. Meganathan, Director

D-cycloserine (DCS) is a naturally occurring broad-spectrum antibiotic produced by *Streptomyces orchidaceus.* It is a structural analog of D-alanine and it is used for the treatment of antibiotic-resistant *Mycobacterium tuberculosis* in conjunction with other first-line drugs like isoniazid and rifampin*.* It inhibits the synthesis of the cell wall by inhibiting the enzymes alanine racemase and D-alanyl-D-alanine ligase. In previous studies, three DCS-resistant *Escherichia coli* mutants were isolated by Curtiss *et al.* (1965). Wargel *et al.* (1971) identified in these mutants that cycA is responsible for the transport of DCS. Later, a study by Baisa *et al.* (2013) reported that cycA is responsible for the transport of DCS only when grown in minimal media but not in complex media. They also further stated that a fully functioning respiratory chain is required for sensitivity to DCS. Ubiquinone mutants, *ubiE, ubiF, ubiG, ubiH* and *ubiX,* were reported resistant to DCS in complex media. In this study, we tested the wild-type and ubiquinone mutants (Δ*ubiB,* Δ*ubiD,* Δ*ubiE,* Δ*ubiF,* Δ*ubiG,* Δ*ubiH,* Δ*ubiH Operon,* Δ*ubiX, and* Δ*ubiI)* in different media and under aerobic and anaerobic conditions. Our studies showed that certain mutants were sensitive to DCS under aerobic and anaerobic conditions in complex media,

whereas all the mutants were sensitive to DCS in aerobic and anaerobic conditions in minimal media with glucose or glycerol as carbon sources. Further, with the oxidizable carbon source glycerol and fumarate or nitrate as electron acceptor, all the mutants were sensitive.

NORTHERN ILLINOIS UNIVERSITY
DE KALB, ILLINOIS

DECEMBER 2017

# EFFECT OF NUTRITION AND GROWTH CONDITIONS ON D-CYCLOSERINE SENSITIVITY OF *ESCHERICHIA COLI*

BY

POORNIMA VASIREDDY
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A THESIS SUBMITTED TO THE GRADUATE SCHOOL

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE

MASTER OF SCIENCE

DEPARTMENT OF BIOLOGICAL SCIENCES

Thesis Director:
Dr. R. Meganathan

ACKNOWLEDGEMENTS

I am very grateful to my advisor, Dr. R. Meganathan, for taking me into his lab and giving me the opportunity to investigate in this area. I am also grateful to my committee members, Dr. Shengde Zhou and Dr. Yanbin Yin, for agreeing to serve on my committee and their valuable suggestions. I would also like to thank Dr. Barrie Bode and the late Dr. Sims for their encouragement and support. Last but not the least, I am very thankful to Patrick McCarthy, who let me borrow materials from him in case of emergency.

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# CHAPTER 1

# INTRODUCTION

D-cycloserine (DCS) is a broad-spectrum antibiotic that is produced by *Streptomyces garyphalus* and *S. lavendulae*. It was first isolated in 1954 as a product of fermentation from *Streptomyces orchidaceus*. Due to its side effects, DCS is rarely used. However, with the emergence of drug-resistant *Mycobacterium tuberculosis*, it is used in combination with other first-line drugs. DCS is either bactericidal or bacteriostatic depending upon the concentration used.

DCS is a cyclic structural analog of L-alanine and D-alanine (Figure 1). Due to its structural similarity, it inhibits cell wall biosynthesis. The cell wall of *Escherichia coli* is made up of *N*-acetylglucosamine and *N*- acetylmuramyl pentapeptide (Figure 2). As seen in the figure the pentapeptide consists of L-alanine-D-glutamic acid- *meso* diaminopimelic acid (mDAP)- D-alanine-D-alanine. The two D-alanines in the terminal end are added as a dipeptide. Two enzymes are required for the biosynthesis of the dipeptide. The enzyme alanine racemase converts L-alanine to D-alanine. A second enzyme, D-alanine ligase, ligates the two D-alanines into the dipeptide D-alanyl-D-alanine. DCS inhibits both alanine racemase and D- alanine ligase (Figure 3).

The DCS-resistant *Escherichia coli* mutants $\chi^{316}$, $\chi^{444}$, and $\chi^{453}$ were first isolated by Curtiss *et al.* in 1965 from a wild-type *E.coli* $\chi^{289}$ by growing in minimal glucose agar medium containing successively higher concentrations of DCS. Wargel *et al.* (1971) identified and

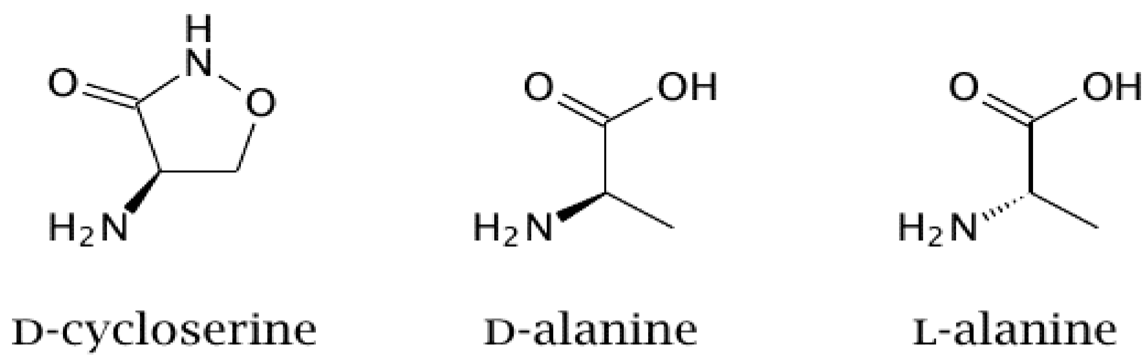


Figure 1: Chemical structures of D-cycloserine, D-alanine, and L-alanine.

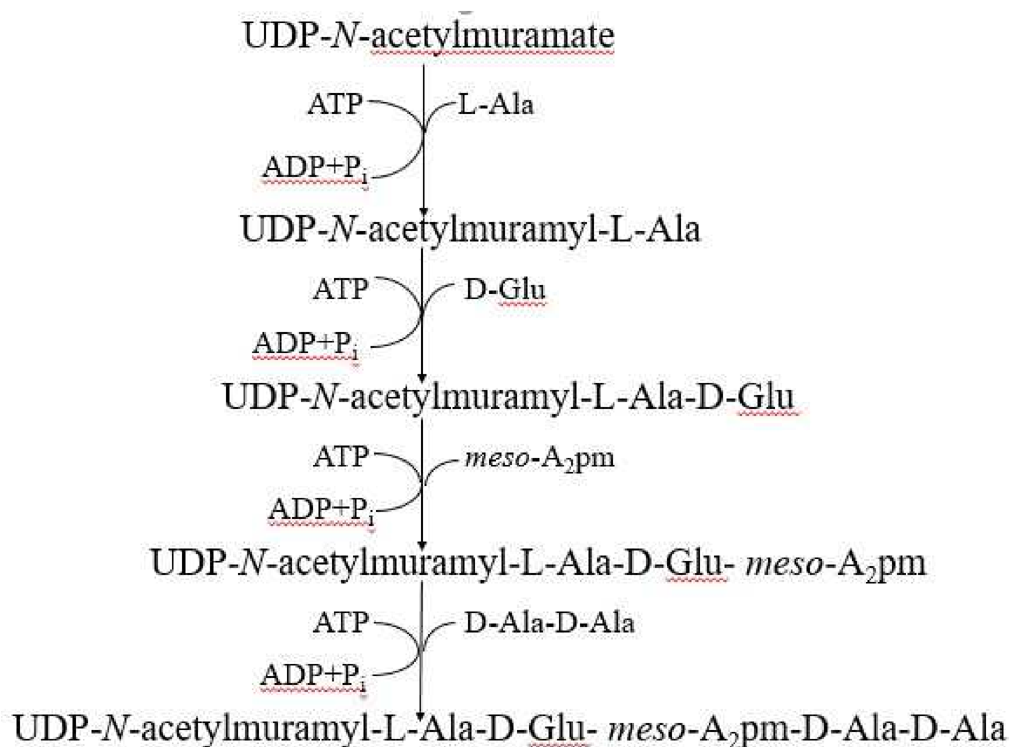


Figure 2: Synthesis of UDP-*N*-acetylmuramyl pentapeptide.

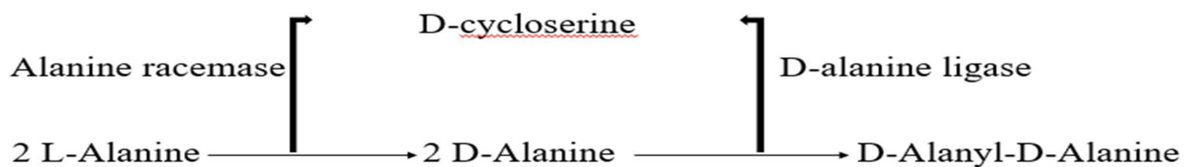


Figure 3: Inhibition of alanine racemase and D-alanine ligase by D-cycloserine.

characterized the role of CycA in the resistance of *E.coli* to DCS growing in minimal media. Baisa *et al.* (2013) identified a set of *E.coli* K-12 mutants from Keio collection *dadA, pnp, ubiE, ubiF, ubiG, ubiH,* and *ubiX* as D-cycloserine-resistant mutants. According to their study, a fully functioning respiratory chain is essential for DCS uptake and sensitivity.

Since E.*coli* is a facultative anaerobe, it can grow aerobically or anaerobically. As discussed above, since DCS is a cell wall biosynthesis inhibitor and *E.coli* being an intestinal organism, we investigated the role of aerobiosis, anaerobiosis and nutrition on DCS resistance and sensitivity using a complete set of deletion mutants blocked in the various steps of ubiquinone (Q) biosynthesis. The Q biosynthetic pathway and the location of the different genes and enzymes are shown in Figure 4.

Q plays a vital role in the electron transport chain and is derived from the shikimate pathway intermediate chorismate. Q synthesis involves nine enzymes encoded by *ubiA, ubiB, ubiC, ubiD, ubiE, ubiF, ubiG, ubiH,* and *ubiX.* The *ubiC* encodes for chorismate pyruvate lyase which converts the chorismate (I) to 4-hydroxybenzoate (II). The 4-hydroxybenzoate (II) is prenylated to 3-octaprenyl-4-hydroxybenzoate (III) by 4- hydroxybenzoate octaprenyl transferase, encoded by the *ubiA* gene. *ubiD* and *ubiX* genes encode decarboxylases which convert 3-octaprenyl-4-hydroxybenzoate (III) to 2-octaprenylphenol (IV). This conversion was demonstrated by Cox *et al.*1969. It was shown that the *ubiD* mutants formed around 20% of the wild-type levels of Q, indicating the mutants are leaky or the presence of another enzyme (Leppik *et al.,* 1976). The decarboxylation is followed by three alternating hydroxylation and methylation reactions.

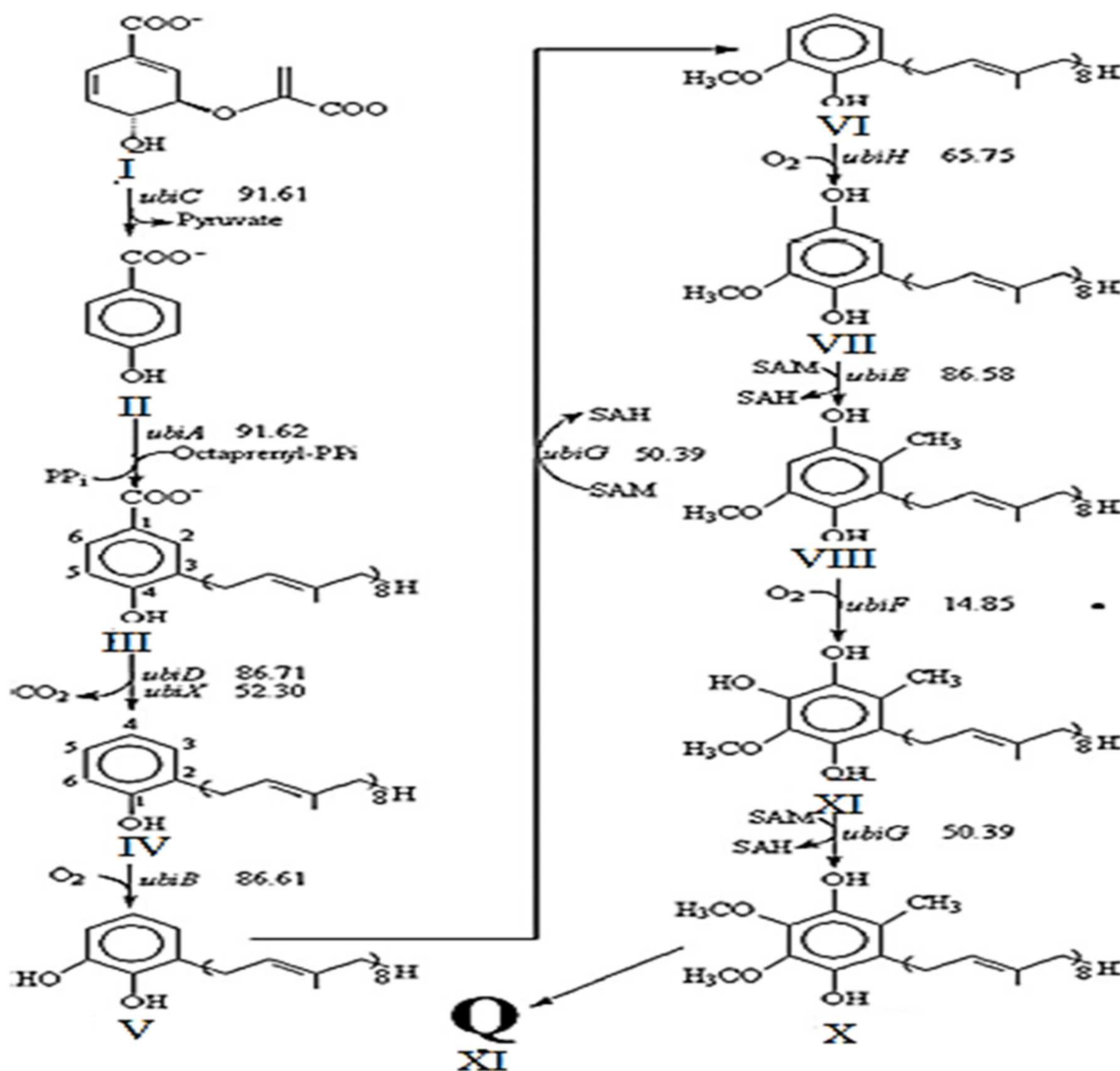


Figure 4: Ubiquinone biosynthesic pathway. The chemical names for the intermediates of the pathway are as follows: (I), chorismate; (II), 4-hydroxybenzoate; (III), 3-octaprenyl-4-hydroxybenzoate; (IV), 2-octaprenylphenol; (V), 2-octaprenyl-6-hydroxyphenol; (VI), 2-octaprenyl-6-methoxyphenol; (VII), 2-octaprenyl-6-methoxy-1,4-benzoquinol; (VIII), 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol; (IX), 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinol; (X), ubiquinol; (XI), ubiquinone (Meganathan & Kwon, 2009).

Three monooxygenases are involved in the introduction of three hydroxyl groups at positions C-6, C-4, and C-5 of the benzene ring, respectively.

1) 2- octaprenylphenol (IV) ⟶ 2-octaprenyl-6-hydroxyphenol (V)
2) 2- octaprenyl-6-methoxyphenol (VII) ⟶ 2-octaprenyl-6-methoxy-1,4-benzoquinol (VIII)
3) 2- octaprenyl-3-methyl-6-methoxy-1,4- benzoquinol (IX) ⟶ 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinol (X)

The three methylation reactions of the pathway, of which two are on *O* and one is on *C*.

These reactions are as follows:

1) 2-octaprenyl-6-hydroxyphenol (V)⟶ 2-octaprenyl-6-methoxyphenol (VI) (i.e O-methylation)
2) 2-octaprenyl-6methoxy-1,4-benzoquinol(VII) ⟶ 2-octaprenyl-3-methy-6-methoxy-1,4-benzoquinol(VIII) (i.e C-methylation)
3) 2-octaprenyl-3-methl-5hydroxy-6methoxy-1,4-benzoquinol(IX) ⟶ ubiquinol (X) (i.e O-methylation)

As mentioned above, the three methylations alternate with hydroxylations, introducing methyl groups at the 6-OH, at the ring C-3, and at the 5-OH respectively. *S*- adenosylmethionine (SAM) is the actual methyl donor, which is converted to *S*- adenosylhomocysteine (SAH). A non-specific *C*- methyltransferase encoded by *ubiE* gene methylates the ring C-3 resulting in the conversion of (VII) – (VIII). In addition, this methyltransferase also methylates dimethylmenaquinone to menaquinone. Both *O*- methylations mentioned above, namely the

methylation of 6-OH and 5-OH, are carried out by the same *O*- methyltransferase encoded by *ubiG* gene (Hsu *et al.,* 1996).

The mutants blocked in the aerobic hydroxylation reactions described above could synthesize 50-70% wild-type levels of ubiquinone when grown anaerobically in glycerol fumarate media. Anaerobically these mutants use hydroxylases to incorporate hydroxyl groups derived from water (Alexander & Young, 1978). However, the genes encoding these hydroxylases remain unidentified. It should be pointed out that a recent paper reports that *ubiB* gene is a regulatory gene and claims that the actual hydroxylation is carried out by *visC* (*ubiI*) gene (Chehade *et al.,* 2013).

This study determines the role of *ubi* mutants and the effect of nutrition on DCS sensitivity of *E.coli*. It is well established that as a facultative anaerobe *E.coli* lives in the intestinal tract under microaerophilic and anaerobic conditions. In the natural environment, the presence of respiratory chain is irrelevant. Hence, we investigated the effect of DCS on *E.coli* and its *ubi* mutants under various nutritional conditions such as complex media, minimal media with fermentable or oxidizable carbon sources, with alternate electron acceptors and supplementation with D- or L-alanine.

The results obtained with this study will be applicable to other anaerobes, microaerophiles and facultative organisms. Similar studies should be conducted in the future with other antibiotics.

# CHAPTER 2

# MATERIALS AND METHODS

## Bacterial Strains

The wild-type and mutant strains of *E. coli* used in this study are listed in Table 1.

## Media

The stock cultures were stored in glycerated Luria Bertani (LB) broth at -80$^0$ C. Cultures were grown either in complex medium or minimal medium. LB was used as the complex medium. The minimal medium described by Spencer and Guest (1973) or MOPS minimal medium described by Neidhardt *et al.* (1974) was used. Glucose 17mM and glycerol 55mM were used as carbon sources. Nitrate 40mM or fumarate 40mM were used as electron acceptors with glycerol as carbon source. For anaerobic growth, the minimal medium was supplemented with 0.1% casamino acids. Glucose was added to the LB medium where indicated; 5.61mM of L-alanine or D-alanine was added to LB and minimal media plates whenever required. The alanine solutions were filter sterilized with 0.2µm filters. Agar was added at a concentration of 1.8% for solid media.

Table 1

List of Bacterial Strains

| Strain | Relevant characteristics | Source |
| --- | --- | --- |
| BW25113 | F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), $\lambda^-$, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514* | Coli Genetic Stock Center (CGSC) |
| JW2308-4 | Δ*ubiX::kan* | CGSC |
| JW2875 | Δ*ubiH::kan* | CGSC |
| JW0659 | Δ*ubiF::kan* | CGSC |
| JW5581 | Δ*ubiE::kan* | CGSC |
| JW226-1 | Δ*ubiG::kan* | CGSC |
| MK203 | Δ*ubiB::kan* deletion mutant of BW25113 | Laboratory stock |
| MG301 | Δ*ubiD::kan* deletion mutant of BW25113 | Laboratory stock |
| MG302 | Δ*visC::kan* deletion mutant of BW25113 | Laboratory stock |
| MG303 | Δ*ygfB*Δ*pepP*Δ*ubiH*Δ*visC::kan* deletion mutant of BW25113 | Laboratory stock |

## Growth Conditions

Cultures were grown at 37$^{o}$C on LB plates for 12 to 16 hours and on minimal media plates for 24 to 26 hours. Aerobic liquid cultures were grown in 10 mL test tubes with shaking at 37$^{o}$C. Anaerobic broth cultures were grown in screw-capped tubes (13 x 100mm). Anaerobic plates were grown using BD Gaspak$^{TM}$ system.

## Antibiotics

Fresh stocks of antibiotics were prepared, and the solutions were filter sterilized using a 20-µm filter. The various antibiotics used, their concentrations and mode of action are shown in Table 2.

## Antibiotic Sensitivity Assays

The antibiotic sensitivity assays were done as described by Goshu *et al.* (2015).

## Growth Studies in Anaerobic conditions

The cells were grown on LB, LB with glucose and minimal media plates in an anaerobic jar. Seed cultures were grown in LB, LB with glucose and glucose minimal medium as required. The seed cultures were inoculated into tubes to around eight Klett units and incubated at 37$^{o}$C. The readings were taken using a Klett meter with red filter (wavelength 640-700nm).

Table 2

Antibiotics Used and Their Mode of Action

| **Classification** | **Antibiotic** | **Concentration** | **Mode of Action** |
|---|---|---|---|
| **Macrolides** | Erythromycin | 1.5mg/mL | It is a macrolide that reversibly binds to the 50S bacterial ribosome and inhibits protein synthesis. |
| **Aminoglycosides** | Gentamicin | 1mg/mL | It is chemically classified as aminoglycoside and causes misreading of tRNA by binding to the 30S bacterial ribosomal unit. |
| | Neomycin | 3mg/mL | It is chemically classified as aminoglycoside and causes misreading of tRNA by binding to the 30S bacterial ribosomal unit. |
| | Streptomycin | 1mg/mL | It is chemically classified as aminoglycoside and causes misreading of tRNA by binding to the 30S bacterial ribosomal unit. |
| | Amikacin | 3mg/mL | It is chemically classified as aminoglycoside, and it causes misreading of tRNA by binding to the 30S bacterial ribosomal unit. |
| | Tobramycin | 1mg/mL | It is chemically classified as aminoglycoside and causes misreading of tRNA by binding to the 30S bacterial ribosomal unit. |
| **DNA Gyrase inhibitor** | Nalidixic Acid | 3mg/mL | It is quinolone which binds reversibly to the A subunit of DNA gyrase further inhibiting RNA synthesis. |

(Continued on following page)

Table 2 (Continued)

| | | | |
|---|---|---|---|
| **Cell Wall Synthesis Inhibitor** | D-cycloserine | 2.5mg/mL | It is a broad-spectrum antibiotic which inhibits cell wall synthesis by inhibiting the enzymes L-alanine racemase, D-alanine: D-alanine ligase. |

## Growth in Glucose Minimal Media Under Aerobic Conditions

The wild-type and mutants were streaked on glucose minimal media plates. Single colonies were picked from the plates and transferred into the broth and allowed to grow till they reached an OD of 0.16. Seventy-five microliters of seed culture were transferred into the side-armed flasks, and the readings were taken every one hour using a Klett meter with a red filter and a wavelength of 640- 700nm.

# CHAPTER 3

# RESULTS

## D-cycloserine Sensitivity Assays in Complex Media

The wild type and nine ubiquinone mutants were screened for sensitivity to DCS in different media under aerobic and anaerobic growth conditions. As shown in Table 3, in LB, wild type*, ΔubiX,* and *ΔubiI* were sensitive, and the other mutants were resistant aerobically and anaerobically. When LB was supplemented with glucose, wild type, *ΔubiF, ΔubiX,* and *ΔubiI* were sensitive aerobically while the others were resistant. All the mutants and wild type were sensitive to DCS in LB with glucose under anaerobic conditions.

## In MS Medium with Glucose as a Carbon Source

In glucose minimal media, under aerobic and anaerobic conditions, wild type and all mutants were sensitive to DCS. When the glucose minimal medium was supplemented with either L-alanine or D-alanine, the wild type and all the mutants exhibited complete resistance (Table 4).

Table 3

D-cycloserine Inhibition of Ubiquinone Mutants in Complex Media

| **Media** | ***BW-WT*** | ***ΔubiB*** | ***ΔubiD*** | ***ΔubiE*** | ***ΔubiF*** | ***ΔubiG*** | ***ΔubiH*** | ***ΔubiH Operon*** | ***ΔubiX*** | ***ΔvisC*** |
|---|---|---|---|---|---|---|---|---|---|---|
| LB aerobic | 10 | R | R | R | R | R | R | R | 8 | 10 |
| LB anaerobic | 10 | R | R | R | R | R | R | R | 12 | 10 |
| LB+ Glucose aerobic | 14 | R | R | R | 12 | R | R | R | 15 | 10 |
| LB+ Glucose anaerobic | 10 | 12 | 14 | 12 | 12 | 14 | 11 | 10 | 12 | 10 |

Table 4

D-cycloserine Inhibition of Ubiquinone Mutants in Glucose Minimal Media

| **Media** | ***BW-WT*** | ***ΔubiB*** | ***ΔubiD*** | ***ΔubiE*** | ***ΔubiF*** | ***ΔubiG*** | ***ΔubiH*** | ***ΔubiH Operon*** | ***ΔubiX*** | ***ΔvisC*** |
|---|---|---|---|---|---|---|---|---|---|---|
| MS+ Glucose aerobic | 38 | 14 | 28 | 14 | 24 | 14 | 10 | 8 | 26 | 28 |
| MS+ Glucose anaerobic | 39 | 22 | 16 | 16 | 18 | 17 | 15 | 9 | 16 | 17 |
| MS+ glucose+ D-alanine 5.61mM | R | R | R | R | R | R | R | R | R | R |
| MS + glucose+ L-alanine 5.61mM | R | R | R | R | R | R | R | R | R | R |

## In MS Medium with Oxidizable Carbon Source and Alternate Electron Acceptors

In glycerol minimal medium under aerobic conditions, all the mutants were sensitive to DCS. The wild type and *ΔubiX, ΔubiF,* and *ΔubiI* exhibited a greater zone of inhibition than the other mutants. Under anaerobic conditions with nitrate or fumarate as an electron acceptor, all the mutants were sensitive to DCS (Table 5).

## Aminoglycoside Sensitivity Assays

Aminoglycosides are highly potent, broad-spectrum antibiotics used in the treatment of life-threating infections, especially those caused by Gram-negative bacteria. They primarily act by inhibiting protein synthesis by binding to 16S RNA, which disrupts the cell membrane integrity. According to Gamal *et al.,* (2011) *E.coli* is most resistant to streptomycin and susceptible to amikacin. The *E.coli* and mutants were tested with aminoglycosides like streptomycin, gentamicin, neomycin, tobramycin, and amikacin to see if they behaved alike. Like the DCS assays, the aminoglycosides were tested on different growth media under various growth conditions. As shown in Tables 6 – Table 12, all the mutants were resistant to streptomycin under all conditions. The wild type, *ΔubiX,* and *ΔubiI* were sensitive to neomycin under all the growth conditions and media except in glucose minimal media under anaerobic conditions (Table 11). *ΔubiF* was also sensitive to neomycin in LB with glucose and in minimal media with glycerol. All the mutants were resistant to neomycin when grown in glucose minimal media under anaerobic conditions.

Table 5

D-cycloserine inhibition of ubiquinone mutants in Glycerol Minimal Media

| **Media** | ***BW_WT*** | ***ΔubiB*** | ***ΔubiD*** | ***ΔubiE*** | ***ΔubiF*** | ***ΔubiG*** | ***ΔubiH*** | ***ΔubiH Operon*** | ***ΔubiX*** | ***ΔvisC*** |
|---|---|---|---|---|---|---|---|---|---|---|
| MS+ Glycerol aerobic | 44 | 18 | 16 | 34 | 42 | 12 | 34 | 8 | 54 | 44 |
| MS+ glycerol nitrate anaerobic | 36 | 28 | 30 | 26 | 36 | 24 | 28 | 28 | 32 | 38 |
| MS+ glycerol fumarate anaerobic | 32 | 24 | 28 | 20 | 32 | 24 | 30 | 26 | 32 | 34 |

Table 6

Inhibition of Ubiquinone mutants by different antibiotics in LB agar under aerobic conditions

| | | ***BW-WT*** | ***ΔubiB*** | ***ΔubiD*** | ***ΔubiE*** | ***ΔubiF*** | ***ΔubiG*** | ***ΔubiH*** | ***ΔubiH Operon*** | ***ΔubiX*** | ***ΔvisC*** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Macrolide | Erythromycin | R | R | R | R | R | R | R | R | R | R |
| Aminoglycoside | Gentamicin | 16 | 8 | 10 | 10 | 10 | 10 | 8 | 7 | 12 | 15 |
| | Neomycin | 18 | R | R | R | 12 | R | R | R | 8 | 10 |
| | Streptomycin | 16 | R | R | R | R | R | R | R | 10 | 15 |
| | Amikacin | 16 | 10 | 11 | 10 | 14 | 8 | 8 | 8 | 14 | 16 |
| | Tobramycin | 14 | 8 | 10 | 11 | 12 | 8 | 8 | 8 | 12 | 14 |
| DNA Gyrase Inhibitor | Nalidixic Acid | 18 | 18 | 24 | 20 | 22 | 18 | 26 | 24 | 18 | 16 |

Table 7

Inhibition of Ubiquinone Mutants by Different Antibiotics in LB Agar under Anaerobic Conditions

| | | ***BW_WT*** | ***ΔubiB*** | ***ΔubiD*** | ***ΔubiE*** | ***ΔubiF*** | ***ΔubiG*** | ***ΔubiH*** | ***ΔubiH Operon*** | ***ΔubiX*** | ***ΔvisC*** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Macrolide | Erythromycin | R | R | R | R | R | R | R | R | R | R |
| Aminoglycosides | Gentamicin | R | R | R | R | R | R | R | R | R | R |
| | Neomycin | 10 | R | R | R | 8 | R | R | R | 8 | 10 |
| | Streptomycin | R | R | R | R | R | R | R | R | R | R |
| | Amikacin | 14 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 14 | 12 |
| | Tobramycin | 10 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 12 | 10 |
| DNA Gyrase Inhibitor | Nalidixic Acid | 16 | 16 | 14 | 16 | 14 | 16 | 18 | 16 | 14 | 10 |

Table 8

Inhibition of Ubiquinone Mutants by Different Antibiotics in LB Agar with Glucose Under Aerobic Conditions

| | | ***BW-WT*** | ***ΔubiB*** | ***ΔubiD*** | ***ΔubiE*** | ***ΔubiF*** | ***ΔubiG*** | ***ΔubiH*** | ***UbiH Operon*** | ***ΔubiX*** | ***VisC*** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Macrolide | Erythromycin | R | R | R | R | R | R | R | R | R | R |
| Aminoglycosides | Gentamicin | 10 | R | R | R | R | R | R | R | 8 | 10 |
| | Neomycin | 12 | R | R | R | 10 | R | R | R | 8 | 10 |
| | Streptomycin | 8 | R | R | R | R | R | R | R | 8 | 10 |
| | Amikacin | 16 | 8 | 7 | R | 12 | R | R | R | 12 | 14 |
| | Tobramycin | 14 | 7 | 7 | R | 9 | R | R | R | 12 | 12 |
| DNA Gyrase | Nalidixic Acid | 16 | 18 | 22 | 16 | 14 | 18 | 20 | 18 | 18 | 16 |

Table 9

Inhibition of Ubiquinone Mutants by Different Antibiotics in LB Agar with Glucose under Anaerobic Conditions

| | | ***BW_WT*** | ***ΔubiB*** | ***ΔubiD*** | ***ΔubiE*** | ***ΔubiF*** | ***ΔubiG*** | ***ΔubiH*** | ***ΔubiH Operon*** | ***ΔubiX*** | ***ΔvisC*** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Macrolide | Erythromycin | R | R | R | R | R | R | R | R | R | R |
| Aminoglycosides | Gentamicin | 8 | R | 7 | R | R | R | R | R | 7 | 10 |
| | Neomycin | 12 | R | R | R | 10 | R | R | R | 8 | 10 |
| | Streptomycin | 8 | R | R | R | R | R | R | R | 7 | 10 |
| | Amikacin | 12 | R | R | R | 12 | R | R | R | 10 | 14 |
| | Tobramycin | 10 | R | R | R | 9 | R | R | R | 9 | 12 |
| DNA Gyrase Inhibitor | Nalidixic Acid | 14 | 20 | 20 | 14 | 16 | 16 | 18 | 16 | 14 | 16 |

Table 10

Inhibition of Ubiquinone Mutants by Different Antibiotics in MS Agar with Glucose under Aerobic Conditions

| | | ***BW_WT*** | ***ΔubiB*** | ***ΔubiD*** | ***ΔubiE*** | ***ΔubiF*** | ***ΔubiG*** | ***ΔubiH*** | ***ΔubiH Operon*** | ***ΔubiX*** | ***ΔvisC*** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Macrolide | Erythromycin | R | R | R | R | R | R | R | R | R | R |
| Aminoglycosides | Gentamicin | 14 | 10 | 10 | 14 | 10 | 12 | 10 | 10 | 10 | 14 |
| | Neomycin | 14 | R | R | R | 8 | R | R | R | 8 | 12 |
| | Streptomycin | 8 | 10 | R | R | R | R | R | R | 8 | 8 |
| | Amikacin | 16 | 7 | 10 | 7 | 8 | 8 | 10 | 10 | 16 | 16 |
| | Tobramycin | 14 | 7 | 10 | 12 | 12 | 14 | 16 | 14 | 12 | 18 |
| DNA Gyrase | Nalidixic Acid | 20 | 18 | 16 | 16 | 16 | 14 | 14 | 16 | 20 | 20 |

Table 11

Inhibition of Ubiquinone Mutants by Different Antibiotics in MS with Glucose under Anaerobic Conditions

| | | ***BW_WT*** | ***ΔubiB*** | ***ΔubiD*** | ***ΔubiE*** | ***ΔubiF*** | ***ΔubiG*** | ***ΔubiH*** | ***ΔubiH Operon*** | ***ΔubiX*** | ***ΔvisC*** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Macrolide | Erythromycin | R | R | R | R | R | R | R | R | R | R |
| Aminoglycosides | Gentamicin | 8 | 8 | R | 9 | 10 | 7 | 8 | 8 | 8 | 8 |
| | Neomycin | R | R | R | R | R | R | R | R | R | R |
| | Streptomycin | R | R | R | R | R | R | R | R | R | R |
| | Amikacin | 8 | 8 | 8 | 8 | 8 | 8 | 7 | 7 | 8 | 8 |
| | Tobramycin | 8 | 7 | 8 | 8 | 9 | 8 | 8 | 8 | 8 | 8 |
| DNA Gyrase Inhibitor | Nalidixic Acid | 22 | 22 | 16 | 18 | 22 | 24 | 22 | 25 | 18 | 22 |

Table 12

Inhibition of Ubiquinone Mutants by Different Antibiotics in MS Agar with Glycerol under Aerobic Conditions

| | | ***BW_WT*** | ***ΔubiB*** | ***ΔubiD*** | ***ΔubiE*** | ***ΔubiF*** | ***ΔubiG*** | ***ΔubiH*** | ***ΔubiH Operon*** | ***ΔubiX*** | ***ΔvisC*** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Macrolide | Erythromycin | R | R | R | R | R | R | R | R | R | R |
| Aminoglycosides | Gentamicin | 18 | 10 | 8 | 10 | 14 | 12 | 14 | 15 | 14 | 20 |
| | Neomycin | 14 | R | R | R | 10 | R | R | R | 10 | 14 |
| | Streptomycin | 8 | 8 | 9 | R | R | R | R | R | 8 | 8 |
| | Amikacin | 18 | 10 | 10 | 12 | 14 | 14 | 14 | 12 | 16 | 16 |
| | Tobramycin | 16 | 12 | 10 | 12 | 12 | 14 | 16 | 14 | 12 | 18 |
| DNA Gyrase Inhibitor | Nalidixic Acid | 22 | 16 | 24 | 21 | 18 | 14 | 20 | 16 | 14 | 20 |

Tests with gentamicin in LB showed that the wild-type and mutants were sensitive aerobically and resistant anaerobically (Tables 6 and 7). In LB with glucose, wild-type, *ΔubiX,* and *ΔubiI* were sensitive aerobically and anaerobically (Tables 8 and 9). Wild-type and mutants were sensitive in glucose minimal media and glycerol minimal media (Tables 10-12) The only exception was that *ΔubiD,* which was resistant when grown anaerobically in glucose minimal media (Table 11).

The wild-type and mutants were sensitive to amikacin under all the growth conditions and in all the media (Tables 6-12), whereas in LB with glucose, wild-type, *ΔubiF, ΔubiX,* and *ΔubiI* were sensitive under aerobic and anaerobic conditions (Tables 8 and 9).

All the mutants were sensitive to tobramycin in LB and minimal media aerobically and anaerobically (Tables 6 and 7,10-12). When LB was supplemented with glucose, *ΔubiE, ΔubiG, ΔubiH, and ΔubiH Operon* were resistant to tobramycin aerobically (Table 8). Under anaerobic conditions, wild -type, *ΔubiF, ΔubiX,* and *ΔubiI* were sensitive to tobramycin while all the other mutants were resistant (Table 9).

## Erythromycin Sensitivity Assays

Erythromycin is a macrolide that inhibits bacterial protein synthesis reversibly. It binds to the 50S RNA with a specific target in the 23S ribosomal RNA molecule (Mazzei *et al.,* 1993). The macrolides are used to treat a broad range of infections, and they are mostly bacteriostatic.

When the wild type and mutants were tested with erythromycin under various growth conditions and media, all were resistant.

## Nalidixic Acid Sensitivity Assays

Nalidixic acid is a quinolone, and it binds to DNA reversibly and inhibits RNA synthesis. It has a marked antibacterial activity against Gram -negative bacteria. Goss *et al.* (1964), performed studies on the mechanism of action of nalidixic acid in *E.coli* and proved that it interferes with DNA synthesis. Tables 6 –12 shows the results of nalidixic acid assays. As shown in the tables the wild type and mutants are highly sensitive to nalidixic acid in all the media and all the growth conditions.

## Growth Studies of Wild-Type, *ΔubiX, and ΔubiI* Under Anaerobic Conditions

Figure 5 shows the growth of wild type, *ΔubiX,* and *ΔubiI* in LB. As seen from the figure, there was a lag of two hours, followed by a log of six hours reaching a maximum growth of 121 Klett units. Figure 6 shows growth of wild type, *ΔubiX,* and *ΔubiI* in LB with glucose. The lag was for three hours followed by a five-hour log reaching the highest Klett units of 125. Growth of wild type, *ΔubiX,* and ΔubiI in glucose minimal media had a two-hour lag followed by a log for seven hours as shown in figure 7. The growth studies performed in various media indicated good growth, and so further antibiotic assays were performed anaerobically in those media.

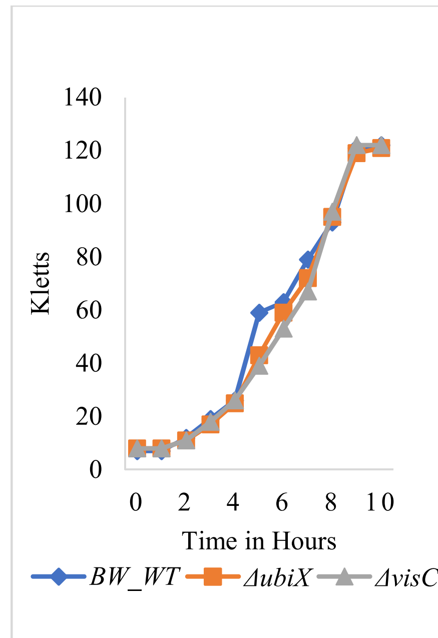


Figure 5: Growth of *BW-WT, ΔubiX,* and *ΔubiI* in LB under anaerobic conditions.

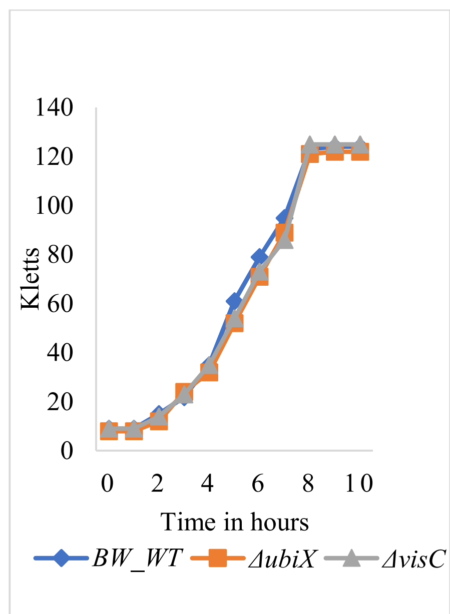


Figure 6: Growth of *BW-WT, ΔubiX,* and *ΔubiI* in LB with glucose under anaerobic conditions.

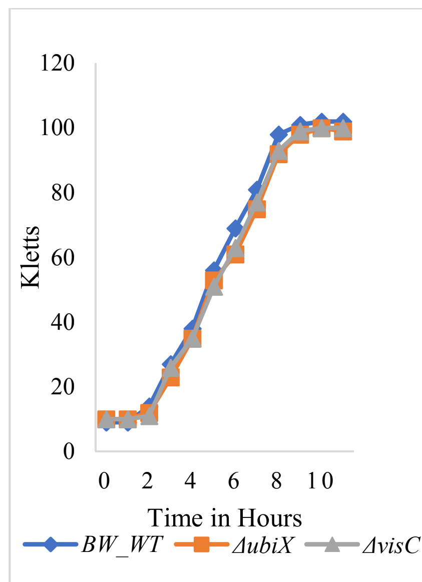


Figure 7: Growth of *BW-WT, ΔubiX,* and *ΔubiI* in glucose minimal medium under anaerobic conditions

<u>Growth Studies of Wild-Type, *ΔubiD, ΔubiX, and ΔubiI* Under Aerobic Conditions</u>

Figure 8 shows growth studies in glucose minimal media. *ΔubiD* and *ΔubiX* had a lag of five hours followed by a log phase for ten hours, whereas the wild type and *ΔubiI* had a lag of six hours followed by a log for eight hours. Figure 9 shows unpublished data from our lab for growth of wild type, *ΔubiD, ΔubiX,* and *ΔubiI* in succinate minimal media.

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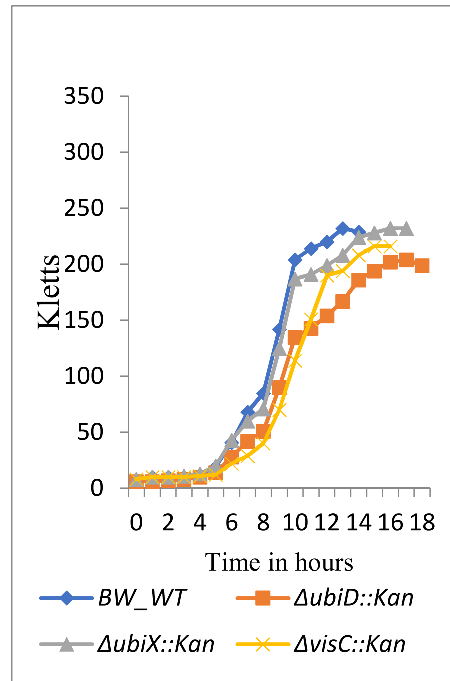


Figure 8: Growth of *BW-WT, ΔubiD, ΔubiX,* and *ΔubiI* in glucose minimal media under aerobic conditions.

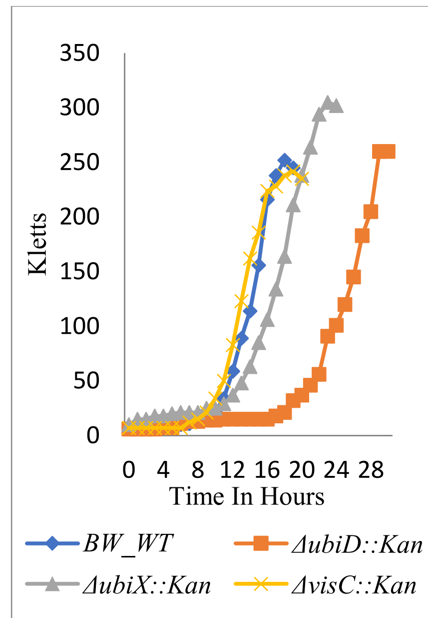


Figure 9: Growth of *BW-WT, ΔubiD, ΔubiX,* and *ΔubiI* in succinate minimal medium under aerobic conditions.

# CHAPTER 4
# DISCUSSION

Baisa *et al.*, 2013 showed that when *E.coli* is grown in complex media the gene cycA is not involved in the transport of DCS. In addition, they stated that the ubi mutants (*ubiE, ubiF, ubiG, ubiH, and ubiX*) from the Keio collection showed resistance to DCS due to the lack of a fully functioning respiratory chain. In this study, we determined the role of the respiratory mutants to DCS sensitivity by using the ubiquinone mutants *ΔubiB, ΔubiD, ΔubiE, ΔubiG, ΔubiH, ΔubiH Operon,* and *ΔubiX,* along with *ΔubiI*, and wild type. The *ΔubiX* mutant can make wild - type levels of ubiquinone in complex and minimal media. *ΔubiI* is not a mutant and behaves like wild type under all conditions.

As discussed previously, the peptidoglycan is made up of UDP-N-acetylmuramic acid and UDP-N- acetylglucosamine which are linked by a β-1, 4 linkage. A pentapeptide L-alanine-D-glutamic acid- *meso* diaminopimelic acid (mDAP)-D-alanine-D-alanine is attached to the UDP-N-acetyl muramic acid. In the synthesis of the pentapeptide, the last two D-alanines are ligated as a dipeptide ( see Fig.2). The conversion of L-alanine to D-alanine is carried out by alanine racemase and a ligase synthesizes the dipeptide D-alanine-D-alanine. A study by Lambert and Neuhaus (1972) showed that the activity of the enzyme alanine racemase was elevated in the cells grown in L-alanine, D-alanine, or pyruvate compared to the cells grown in glucose.

The wild type and mutants were sensitive to DCS when grown in minimal media with glucose or glycerol as carbon source ( seeTable 4). Glucose and glycerol undergo fermentation and oxidation respectively to produce pyruvate. The pyruvate is then aminated to alanine by glutamate pyruvate aminotransferase. Alanine produced from pyruvate forms a pool intracellularly which decreases the alanine racemase activity as the concentration of alanine in the pool is inversely related to the specific activity of alanine racemase (Lambert & Neuhaus, 1972).

Our results from Table 4, show that the DCS resistance of wild type and mutants is due to the conversion L-alanine to D-alanine when grown in glucose minimal media supplemented with alanine. The increased resistance is due to the conversion of L-alanine to D-alanine by alanine racemase as the addition of L-alanine or D-alanine to glucose increases the specific activity of alanine racemase. This in turn promotes the formation of peptidoglycan.

The wild type, *ΔubiX,* and *ΔubiI* were sensitive to DCS when grown in complex media (see Table 3). *E.coli* undergoes diauxic growth when grown in LB medium. After the depletion of available sugars, the cells are forced to utilize the amino acids as carbon sources for metabolism (Sezonov *et al.,* 2007). The LB medium consists of less than 100µM of fermentable sugars and many catabolizable amino acids. L-alanine is present at a concentration of 5.8mM in LB medium. The wild type, *ΔubiX,* and *ΔubiI* have a higher growth rate compared to the other mutants. Hence the sugars and amino acids present in the LB medium are insufficient for the wild type, *ΔubiX,* and *ΔubiI* to prevent the inhibition by DCS. In contrast, sufficient sugars and amino acids are available in the medium for the growth of other mutants. When alanine was added to the LB plain at the concentration of 5.61mM, the increase in the activity of the alanine

racemase made the wild-type and mutants resistant to DCS. The DCS assays showed that the *ΔubiX* and *ΔubiI* behaved like wild type. Hence these mutants were tested for sensitivity to other antibiotics. These results along with growth studies in different media show that *ΔubiI* is not a mutant. As stated before, the UbiD and UbiX produce 80% and 20% of Q respectively. The wild type, *ΔubiD, ΔubiX* and *ΔubiI* mutants were tested for growth on succinate minimal media (unpublished data from our lab). The wild type and *ΔubiI* showed a ten-hour lag. In contrast, the *ΔubiX* mutant showed a 12-hour lag and *ΔubiD* mutant showed a 20-hour lag.

The wild type and mutants were tested for DCS sensitivity by growing them anaerobically on glycerol minimal media with fumarate or nitrate as electron acceptors. All the mutants were sensitive to DCS along with wild -type. The glycerol is converted to pyruvate which is aminated to alanine and is accumulated intracellularly. This intracellular alanine is used as a carbon source and it also decreases the activity of alanine racemase which makes the cells susceptible to DCS.

As mentioned before, *ΔubiB, ΔubiF,* and *ΔubiH* have alternative hydroxylases anaerobically and can make 50 – 60% of wild- type ubiquinone levels (Alexander & Young, 1978). From the results, the *ΔubiB, ΔubiF,* and *ΔubiH* show resistance to DCS when grown under anaerobic conditions in complex media and were sensitive to DCS when grown anaerobically in minimal media. All these observations indicate that there is no involvement of respiratory chain in the DCS sensitivity of *E.coli* but it is dependent on the nutrition.

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