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# Oxygen Limitation Suppresses Reactive Oxygen Species Formation by Norfloxacin

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**Previous research showed that the formation of reactive oxygen species (ROS) serves as a common mechanism of bactericidal antibiotics induced cell death. The purpose of this study was to better understand the mechanism of oxygen radicals formation by assessing the effect of oxygen limitation on the production of ROS when two strains of *Escherichia coli*, BW25113 and JW0419, were treated with the quinolone antibiotic Norfloxacin under aerobic or anaerobic conditions. JW0419 strain is deficient in the *cyoD* gene, a subunit of the cytochrome *bo* terminal oxidase, an aerobic respiratory enzyme predominantly expressed under high oxygen levels. This strain served to assess the ability of cytochrome *bo* to produce oxygen species. Aerobic and anaerobic condition did not affect the minimal inhibitory concentration of norfloxacin for either strain. High concentration of norfloxacin treatment resulted in an increase in ROS formation after one hour of treatment under aerobic conditions for both strains but decreased to a plateau at 2 hours. Anaerobically treated cultures showed a lower steady increase in ROS formation before reaching a plateau at 2 hours. Our data suggests that oxygen was required for norfloxacin induced increase in the formation of oxygen radicals, in a mechanism which is likely independent of cytochrome *bo* activity.**

In addition to the direct drug-target interactions of fluoroquinolones, beta-lactams and aminoglycosides antibiotics, Kohanski *et al.* showed that hydroxyl radical induction is a potential alternative mechanism of bacteria killing common among the three major classes of bactericidal antibiotics (1).Norfloxacin (a fluoroquinolone) inhibits bacterial replication by antagonizing DNA gyrase, an enzyme necessary to separate bacterial DNA strands during cell division (2). It is representative of ROS inducing bactericidal antibiotic killing and is used in this study.

*Escherichia coli* is a facultative anaerobic bacterium. Under aerobic conditions, the carbon source is catabolized and shunted into the tricarboxylic acid cycle and oxygen is used as terminal electron acceptor (TEA), and produces superoxide radicals, which subsequently are converted to $H_2O_2$ and water. In bacteria, a major source of $H_2O_2$ is generated by this mechanism. Under anaerobic conditions, oxygen is not used and no waste products consisting of oxygen species are produced (3).

NADH-coupled electron transport system is essential parts of aerobic respiration and was induced under bactericidal antibiotic treatment (1). Decoupling NADH from ETC by knocking out the TCA components hindered hydroxyl radical formation and bacterial killing (1). Hydroxyl radical formation involved the reduction of $H_2O_2$ by intracellular ferrous iron, and this event coincided with increased catabolic depletion of NADH via respiratory ETC (1). The formation of hydrogen peroxide was confirmed by a measured increase of $H_2O_2$activated promoter activity upon antibiotic treatment, and growth inhibition upon $H_2O_2$ addition (4).

*E. coli* terminal oxido-reductases exhibited differential activities under oxygen abundant and oxygen scarce conditions. Reduction of oxygen can be carried out by one of the three major terminal oxidoreductases: cytochrome-bo (cyt-*bo*), cytochrome-*bd*-I (cyt-*bd*-I) and cytochrome-*bd*-II (cyt-*bd*-II) (5, 6). Cyt-*bo* is preferentially expressed under oxygen rich conditions, with relatively low affinity to substrate, but a high processing rate (5). Besides reduction of oxygen, cytochrome-*bo* contributes two protons during each reduction event to the generating the proton motive force (5). Essential components of the cytochrome include 4 subunits, cyoABCD; deficiency in a single subunit renders the complex non-functional (5). On the other hand, cytochrome-*bd*-I is expressed under low oxygen conditions with a lower processing rate (5, 6). Cyt-*bd*-II uniquely consumes periplasmic $H^+$ in the formation of water so does not contribute to producing the proton gradient (6).

In our study, a *cyoD* deletion mutant strain (*ΔcyoD*), JW0419-1, which is deficient in cyt-*bo* activity and exhibits impairment in oxygen-dependent metabolism, was used to assess its susceptibility to norfloxacin treatment under oxygen abundant and anoxic conditions, as well as to examine the role of *cyt-bo* in the generation of ROS (7, 8). Bacteria demonstrate higher efficiency in metabolic activities and therefore higher rates of growth and uptake when aerobic respiration is feasible, due to ability to generate more ATP. Mutants deficient in cyt-*bo* should exhibit reduced metabolism compared to wildtype in aerated culturing conditions and exhibit similar growth rates under aerobic and anaerobic states due to impaired ability to use oxygen as TEA. Discrepancies between the expectations and actual growth performance are discussed.

## MATERIALS AND METHODS

**Bacterial strains.** JW0419-1*ΔcyoD786::kan* mutant of *Escherichia coli* K-12 from the KEIO collection and the parental strain BW25113, were obtained from the Coli Genetic Stock Center (7).

**Culture and media.** All cultures were incubated at 37°C on shaking platform in M9 salts minimal media, consisting (w/v) of

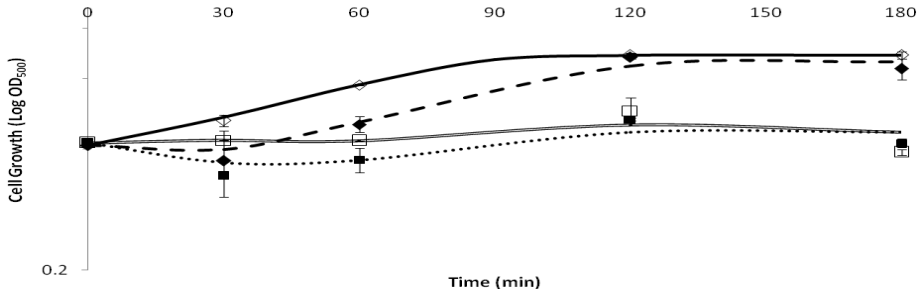


**FIG 1** The effect of anaerobic and aerobic conditions on the growth of *E. coli*BW25113 (parental) incubated with norfloxacin. Norfloxacin is added at 0 minutes. Aerobic, norfloxacin treated(═□═).Anaerobic, norfloxacin treated (··■··).Aerobic, untreated (—◇—). Anaerobic untreated (╾◆╾).

0.05% NaCl, 0.7% $Na_2HPO_4$, 0.3% $KH_2PO_4$, 0.1% $NH_4Cl$, 0.02% $MgSO_4$•$7H_20$, and 0.2% glucose.1X PBS buffer was used to wash and resuspend cell pellets during ROS assay, consisting (w/v) of 0.8% NaCl, 0.02%KCl, 0.144% $Na_2HPO_4$, and 0.024% $KH_2PO_4$. Both media has adjusted pH to 7.3-7.4.

**Preliminary growth curve measurement.** Colonies of each strain were inoculated in M9 minimal salts media and incubated overnight with shaking at 37°C. On the following day, overnight cultures were diluted in additional M9 salts minimal media to an $OD_{500}$ of 0.15. Cultures were divided to be incubated either aerobically in 250ml flasks on 37°C shaking water bath or anaerobically in 13X150mm tubes and in anaerobic jars at 37°C incubator without shaking. In order to minimize the oxygen contamination for anaerobic cultures during turbidity reading, one anaerobic jar was prepared for each time points. At each time point, the samples were taken out for turbidity reading at 500nm using Spectrometer 20D.

**Minimum inhibitory concentration (MIC) assay.** Overnight cultures of each E coli strain were added in duplicate to a 96-well microtitre plate containing norfloxacin (4.8 to 0.004µg/ml in serial dilutions with a dilution factor of 2), and a positive control containing no antibiotic. Sterile M9 salts minimal media was added to empty wells as a negative control. The plate was incubated overnight at 37°C, and the MIC was established as the lowest concentration of antibiotic at which there was no visible growth (9).

**Cell growth assay with antibiotics**. Overnight cultures of two *E. coli* strains were diluted 1/10 with M9 salts minimal media and incubated at 37°C anaerobically for 3-4 hours. After pre-incubation, cell cultures were diluted in M9 salts minimal media to reach an $OD_{500}$ of 0.5. The norfloxacin was then added in the cell cultures to a final concentration of 20 µg/ml. The cultures were then immediately split and incubated at 37°C under aerobic and anaerobic conditions. At each time point, 150 µl of cell cultures from both aerobic condition and anaerobic condition were added in duplicate to a 96-well microtitre plate (Falcon) containing an equal volume of M9 salts minimal media. The 96-well plate was kept on ice to inhibit bacteria growth. After collecting cell cultures from all time points, the turbidity of the 96-well microtitre plate wells was read at 500nm.

**Detection and Measurement of Reactive Oxygen Species (ROS).**Overnight cultures of two *E. coli* strains were diluted 1/10 with M9 salts minimal media and incubated at 37°C anaerobically for 3-4 hours. After pre-incubation, cell cultures were diluted in M9 salts minimal media to reach an $OD_{500}$ of 0.5. The norfloxacin was then added in the cell cultures to a final concentration of 20 µg/ml. The cultures were then immediately split and incubated at 37°C under aerobic and anaerobic conditions. At each time points, 3 ml in duplicate of each cell cultures containing norfloxacin, along with a negative control that contained cell culture only were centrifuged for 1 min at 13,400 x g. Cell pellets were resuspended with 1ml of 1X PBS. For cell cultures of anaerobic condition, anaerobic PBS solution was made by bubbling with 5% $CO_2$, 10% $H_2$ and 85% $N_2$ gas mildly for 5min in order to eliminate oxygen dissolved in the PBS solution. After resuspension, cell cultures were treated with 1µl of 2',7'-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) reagent (Sigma-Aldrich) and incubated in dark for 20min. Cell cultures were then centrifuged for 1 min at 12,000rpm after incubation with $H_2$DCFDA reagent. Cell pellets were then washed with 1 ml 1 X PBS and spun down again. Pellets were resuspended in 1ml 1X PBS, and the solution was transferred to a 2 ml glass bead tube. The samples were homogenized with Fastprep homogenizer with settings of 6.5 m/s for 3 runs of 30 sec with 5 min cooling between runs. Cell lysates were centrifuged for 1 min at 13,400x g to remove debris and beads, and the fluorescence was read in a Turner fluorometer.

**Gas displacement of anaerobic jar.** Metal anaerobic jars were used in these experiments in order to create an anaerobic environment, and all anaerobic tanks were pre-warmed to 37°C before being loaded with cell cultures. In order to replace the gas in the tanks, the sealed tanks were vacuumed, followed by gassing with 5% $CO_2$, 10% $H_2$ and 85% $N_2$. The process was repeated three times to ensure a total gas replacement.

## RESULTS

**The presence of oxygen in culturing conditions did not affect bacteriostatic effect of norfloxacin.** The MIC determined in this study for both strains under aerobic and anaerobic conditions are 4.8 µg/ml. This is significantly higher than theoretical MIC obtained from previous research, which ranged from 0.04 to 0.06 µg/ml (9, 10). This unexpected result demonstrated that oxygen limitation does not reduce the bacteriostatic effect of norfloxacin at the inhibitory concentration.

**Norfloxacin inhibited cell proliferation as treated cells were not dividing.** Microscopic evaluation showed that in contrast to the untreated cells, no dividing cells were observed in norfloxacin-treated cultures in aerobic or anaerobic conditions (data not shown). No observable

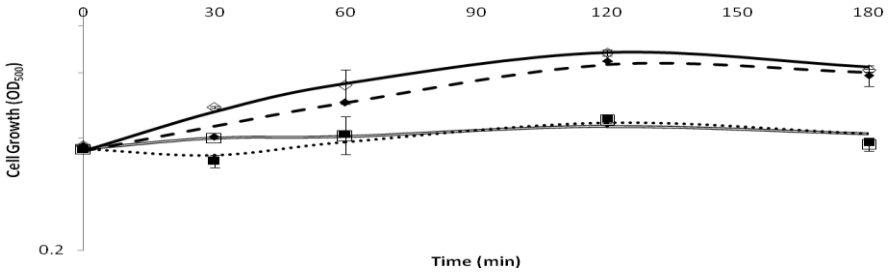


**FIG 2** The effect of anaerobic and aerobic conditions on the growth of *E. coli* JW0419 (*ΔcyoD*) incubated with norfloxacin. Norfloxacin is added at 0 minutes. Aerobic, norfloxacin treated (=□=). Anaerobic, norfloxacin treated (▪▪■▪▪). Aerobic, untreated (—◇—). Anaerobic untreated (▬◆▬).

difference in size or morphology was evident between treated or untreated samples at 60 minutes and 120 minutes after initiation of treatment. Plate counts were not performed since plating step exposes the bacteria to air where the presence of oxygen could induce hydroxyl radical-mediated killing, and cells could also have taken up norfloxacin and died in culture after being spread plated.

**Presence of oxygen did not affect growth rates of either strain.** Upon treatment of norfloxacin, the turbidity of both strains remained relatively constant under aerobic condition and showed an immediate 10% decrease in growth under anaerobic condition (Fig. 1, 2). The untreated cultures grew at the same rate with or without oxygen until plateau by 120 minutes and might have decreased 10% or 15%, with an unclear trend of a prolonged lag for both anaerobic untreated cultures (Fig. 1, 2). Since the overall extent of growth and growth rate seemed to not differ greatly under aerobic and anaerobic conditions (Fig. 1, 2), it appears as though both strains grew independent of aerobic metabolism. The parental and mutant strains showed no obvious differences in growth either (Fig. 1, 2), suggesting the mutation's effect on growth is limited.

**Hydroxyl radical formation increased in aerobic norfloxacin treated cultures.** In the presence of $O_2$, both strains treated with norfloxacin had a significant increase in fluorescence emission at time 60 minutes but then dropped down for the next 2 hours (Fig. 3, 4). The fluorescence of the untreated aerobic control cultures remained roughly constant over the whole period. Comparing the absolute values of the specific fluorescence, the mutant strain had higher fluorescence generated over the time course than its parental strain, and a slight decrease in the fluorescence reading after 30 minutes of norfloxacin treatment was observed in the mutant strain which was not shown in the wild type strain.

**Under anaerobic condition, fluorescence increased after 60 minutes in the parental culture but not in the *ΔcyoD* culture.** The fluorescence measuring ROS level of the parental strain steadily increased during the 180 minutes time but was 2 times lower than the peak at the 60 minutes under aerobic condition before leveling to similar levels at 120 minutes (Fig. 3). The fluorescence reading for the *ΔcyoD* strain had no significant change when $O_2$ was limited, and was considerably lower than the aerobic fluorescence readings. The difference in ROS induction may be attributed to the lack of cytochrome-*bo* activity.

## DISCUSSION

The knockout of *cyoD* gene and the resulting non-functional cytochrome-*bo* terminal oxidase renders the mutant unable to respire aerobically catalyzing the oxidation of ubiquinol and reduction of oxygen in the ETC under oxygen rich condition. However, *cyo* operon expression is found to be the lowest when glucose is the only carbon source in the medium compared to otherunfermentable carbon sources (11). This might explain the unexpected growth curve of the *cyoD* deletion mutant which is similar to the parental strain under both conditions in the sense of growth rate and doubling time. Both strains are likely to use substrate level phosphorylation as the major metabolism process as aerobic respiration is affected by the carbon source regulation even when oxygen is present, independent of the functioning of cytochrome-*bo* terminal oxidase. Since the other cytochrome-*bd-I* terminal oxidase is moderately expressed aerobically, some aerobic respiration is still expected for both strains under aerobic conditions (10). When oxygen was limited, both strains showed a lag of growth which is expected as time is needed to switch to cellular machinery expressed in anaerobic environment, again observed in the turbidity changes of negative controls during ROS level measurements (Fig. 1, 2). Growth rates under anaerobic condition were also slightly lower than aerobic condition as expected since aerobic respiration using oxygen as TEA was completely switched off.

The bacteriostatic activities of norfloxacin are similar under both aerobic and anaerobic conditions. Quinolones, unlike aminoglycosides, do not require oxygen for the uptake of the antibiotic into the cell. Our results were consistent with the findings by Malik *et al.* (2007)in a study on quinolone lethality under aerobic and anaerobic conditions (12). The study noted that in *E. coli*, norfloxacin required higher concentrations of antibiotic for bactericidal activity in anaerobic conditions than in aerobic

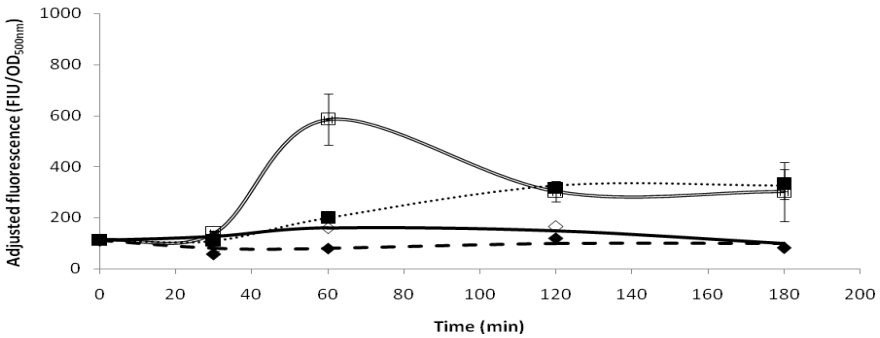


**FIG 3** The level of ROS reflected by $H_2$DCFDA fluorescence at 515nm in *E. coli*BW25113 (parental) after treatment by norfloxacin. The antibiotic was added at time 0, and is compared with untreated control under aerobic and anaerobic conditions. The fluorescence reading was adjusted by total cell density to account for the changes in total fluorescence due to increased cell density. Aerobic, norfloxacin treated (═□═).Anaerobic, norfloxacin treated (▪▪▪). Aerobic, untreated (◇). Anaerobic untreated (▬◆▬).

conditions (12).Furthermore, their MIC values were 0.075 µg/ml for norfloxacin with an observed bactericidal effect for aerobic conditions that was logarithmically exponential from 1X MIC to 10X MIC where at 10X MIC the effect plateauing. For anaerobic conditions, the same trend was seen but started at 10X MIC instead (12). The MIC of our strains were not as expected, with publications detailing MICs of 0.025 to 0.075µg/ml for *E. coli* (12, 10). A likely explanation is that the media used affected the uptake of norfloxacin. In the publications with lower MICs, Luria broth (LB) was used in contrast to our use of M9 minimal medium. It was discovered that increasing concentrations of magnesium negatively affect the antibacterial potency of norfloxacin and other quinolones (13). This phenomenon is due to magnesium ions (and other alkaline earth metal ions) binding directly to the oxygen atoms found on two molecules of norfloxacin and creating a dimeric complex (14). The interaction results in an increase in solubility in water and decreased ability to diffuse into the cell (14). It is probable that magnesium played a factor in the differences as M9 minimal medium contains more than 20 times the concentration of magnesium ions as LB. Our treatment condition had a $Mg^{2+}$/norfloxacin ratio of 10,000, which was shown to increase the MIC of certain strains of *E. coli* to more than 100-fold (15). Whether this complex formation affects the intracellular activity of the antibacterial or if it affects ROS production is unknown.

Sangurdekar *et al.* (2006) performed a microarray analysis on antibiotic treated *E. coli* to look for genes affected by treatment with norfloxacin and found that the antibiotic affected the transcription levels of anaerobic genes and targets of the fumarate/nitrate reductase (FNR), a transcriptional regulator that facilitates growth transition between aerobic and anaerobic conditions (16). This change in gene expression could result in the increase of FNR regulated fumarate reductase in norfloxacin-treated cultures. On the other hand, although pre-cultures were inoculated anaerobically prior to the initiation of the assay, anaerobic cultures continue to adapt to anoxic environment and express enzymes relevant to anaerobic metabolism, such as fumarate reductase, independent of the effect of norfloxacin. Fumarate reductase exhibits intrinsic redox potential to reduce oxygen, forming superoxide which can be subsequently converted to hydrogen peroxide and hydroxyl radicals. Although no terminal oxygen reductase activity was expected during the anaerobic incubation, the short period of sample processing at the termination of the time points provided an opportunity for oxygen contact that might shift the cultures rapidly from anaerobic to an aerobic system. This brief exposure to oxygen would cause fumarate reductase to rapidly produce superoxide (17). Hence, this mechanism could explain why there was detection of ROS in the untreated anaerobic culture but greater levels of ROS detected in the norfloxacin-treated anaerobic culture. Yet, in our anaerobic condition where the parental cells were treated with norfloxacin, we see an increase in ROS detection that is similar to aerobic conditions. This may be due to a summative effect of having cyt-*bo* as well as fumarate reductase, thereby producing a greater amount of ROS once exposed to air during sampling (17).

Contrary to prior published data (1), our data showed a lack of difference in fluorescence after three hours of norfloxacin treatment compared with control culture. Based on the finding that increasing norfloxacin concentration above the MIC reduced detectable amount of ROS generated by *E. coli*, while bacteria killing was unaffected, one may suspect that a similar mechanism might play a role in our result. As previously discussed, the high concentration of norfloxacin selected for use in our assay (due to quenching effect by the magnesium in the media) was eight times higher than the concentration in which the effect of equalization in the ROS level between treated and untreated cultures began to establish(12). Therefore, the level of ROS induced may not proportionally correspond with the antibiotic concentration and the extent of cell killing.

The amount of ROS showed rapid increase at 30 min in norfloxacin treated cultures of both strains under aerobic conditions then diminished to a plateau in the following two hours (Fig. 3, 4). A study by Liu *et al*. showed that

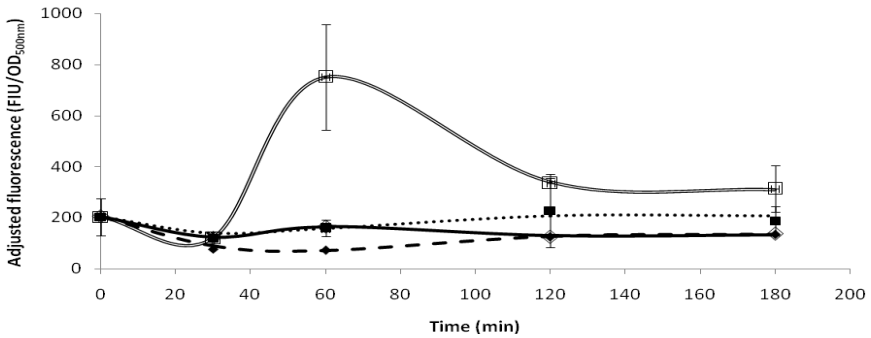


**FIG 4** The level of ROS reflected by $H_2$DCFDA fluorescence at 515 nm in *E. coli* JW0419 (*ΔcyoD*) after treatment by norfloxacin. The antibiotic was added at time 0, and is compared with untreated control under aerobic and anaerobic conditions. The fluorescence reading was adjusted by total cell density to account for the changes in total fluorescence due to increased cell density. Aerobic, norfloxacin treated (═□═).Anaerobic, norfloxacin treated (▪▪■▪▪). Aerobic, untreated (◇). Anaerobic untreated (▬◆▬).

there was increasing induction of ROS formation starting at 1 h and peaking at 2 h and a slight drop was seen at 3h (18). The trend in Liu's data is similar to ours except our induction started at 30 min, which brought into speculation that perhaps the time of induction is affected by some other factor(s). This factor could be due to the time lag in splitting cultures after the addition of norfloxacin and our actual reference "time zero" of incubation. The difference in fluorescence signal between *ΔcyoD* strain and the parental strain could be due to background caused due to sampling.

In conclusion, our results showed that ROS formation was reduced in the absence of oxygen. Ergo, there is a requirement for oxygen by the bactericidal antibiotic, norfloxacin, for the formation of ROS. The use of the *ΔcyoD* strain, in comparison with the parental strain, demonstrated similar ROS induction and suggested the lack of direct involvement of cytochrome-*bo* in norfloxacin induced hydroxyl radical formation.

## FUTURE DIRECTIONS

Due to limitations in equipment, a true anaerobic analysis was difficult. Alternatives are to use antagonists to inhibit or uncouple the ETC, or use mutants with genetic defects in the ETC. A particular E. coli strain, designated as MB44, has all three terminal oxidases knocked out thereby preventing the consumption of oxygen and shifts the bacteria to use homolactic fermentation for ATP production (8). The use of this strain would be particularly useful for assessing and deriving other sources of reactive oxygen species, if any. Modifying the composition of the minimal medium by adjusting the final concentration of magnesium ion and carbon source would also reveal additional information on the effect of norfloxacin on ROS level, by removing the interference of high $Mg^{2+}$ ions and the down-regulation of aerobic respiratory enzymes by glucose.

The potential pathway for superoxide formation should be tested by using double knockout strains with deficiencies in combinations of the cytochrome-*bd*-I, *bd*-II and cytochrome-*bo*. This test would allow us to understand whether each cytochrome alone could be the source the ROS induced by the bactericidal antibiotics.

Our preliminary experiment demonstrated the ROS formation induced by ampicillin treatment under anaerobic condition was earlier and considerably higher than norfloxacin treatment (data not shown) and further study of ROS formation induced by ampicillin can also give insights into the ROS induced cell killing mechanism by different classes of antibiotics.

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