Deciphering the role of oxidative stress response in bactericidal antibiotic resistance

A Thesis

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by

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Certificate

This is to certify that this dissertation entitled “Deciphering the role of oxidative stress response in bactericidal antibiotic resistance” towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by V. K. Sirisha at Indian Institute of Science Education and Research under the supervision of Dr. Sunish Radhakrishnan, Associate Professor, Department of Biology, during the academic year 2020-2021.

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Date: 18.05.2021

This thesis is dedicated to Dr. Sailusha Vadapalli

Declaration

I hereby declare that the matter embodied in the report entitled "Deciphering the role of oxidative stress response in bactericidal antibiotic resistance" are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of Dr. Sunish Radhakrishnan and the same has not been submitted elsewhere for any other degree.

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Date: 18.05.2021

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Abstract

In addition to acting on the primary targets, recent evidence indicates that bactericidal antibiotics such as kanamycin, norfloxacin, ampicillin have the ability to induce oxidative stress in bacterial cells, which in turn may increase the killing efficacy of the antibiotics. However, it remains to be understood if the oxidative stress response mechanisms in bacteria play any role in enhancing the resistance of bacterial cells towards the antibiotic. The oxidative stress response regulator, OxyR, plays a major role in overcoming the oxidative stress response in bacteria. Using a gain-of-function mutant of OxyR from *Caulobacter crescentus*, this work attempts to dissect the mechanism and role of OxyR during antibiotic-induced oxidative stress.

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Finally, without the constant support and love from my friends and family, this fulfilling yet arduous time would have been incredibly difficult.

Introduction

Humans are at a constant exposure to bacteria whether they be beneficial, harmless or pathogenic. Even with all the recent advancements in modern medicine and the development of antibiotics, bacterial infections are the cause for 0.01% of all deaths worldwide [29]. Antimicrobials have been instrumental in the progress made in medical sciences and have been in use since the discovery of penicillin in 1928. Since then, many mechanisms of resistance have also been discovered [30]. Due to misuse and overuse of antimicrobials over the years, the emergence of multidrug resistant pathogens is becoming more and more common.

Antibiotics: Classes and mechanisms of action

To understand how antibiotic resistance develops, it is important to understand how antibiotics work. Antibiotics can either stop the growth of bacteria (bacteriostatic) or may cause cell death (bactericidal). Based on the mechanism of action, or the cellular target of the drugs, antibiotics can be grouped into the following groups [1,2] (**Figure1**):

- Protein synthesis inhibitors
    - 30S subunit inhibitors
        - Aminoglycosides: these have a bactericidal effect by inhibiting protein synthesis by targeting ribosome binding with DNA thus inhibiting translational initiation and elongation leading to cell death.
        - Tetracyclines: these have a bacteriostatic effect by inhibition of aminoacyl tRNA binding to the ribosomal complex.
    - 50S subunit inhibitors
        - Peptidyl transferase inhibitors: these have a bacteriostatic effect by inhibiting peptide bond formation in the ribosomal complex.
        - MLS antibiotics: these groups of bacteriostatic antibiotics, though structurally different, act by blocking the path through which nascent peptides exit the ribosomal complex thus dissociating the peptidyl tRNA from the ribosome [32].
- Cell wall synthesis inhibitors
    - β- lactams: They have a bactericidal effect as they structurally resemble the short peptides of peptidoglycan that interact with penicillin binding proteins and competitively inhibit PBPs from cross-linking the short peptides thus inhibiting cell wall synthesis [28].
    - Glycopeptides: They have a bactericidal effect by inhibiting transpeptidase which is required for formation of cross-links between different layers of peptidoglycan resulting in weak cell walls that easily lyse under the cell's internal pressure.
- Nucleic acid synthesis inhibitors
    - Quinolones have bactericidal effects by affecting the chromosomal topology by binding to topoisomerases and gyrases thereby hindering the segregation of replicated daughter chromosomes or inhibiting the release of supercoiling resulting from transcriptional and replication complexes moving along DNA, thus causing great stress and cell death.[33].

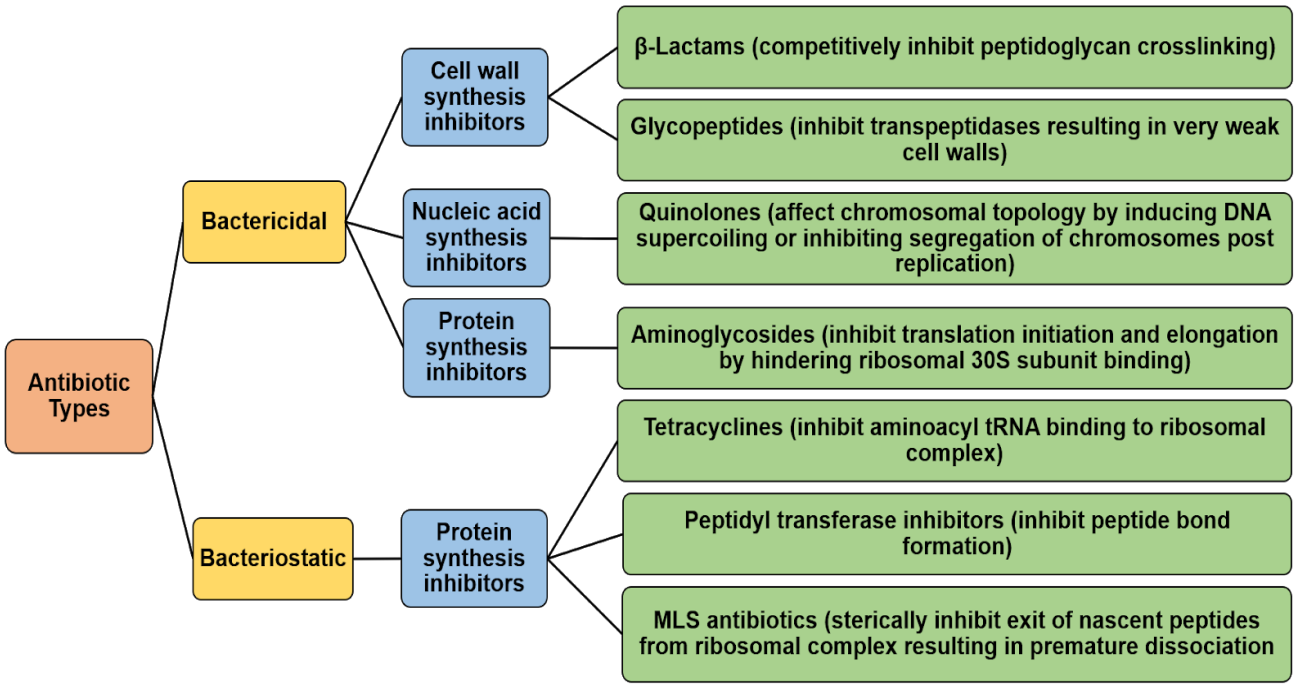

Figure 1: Antibiotic classification based on activity and mechanism of action.

Though the drug target interactions are fairly well known, the bacterial responses to the antibiotics are not understood so well. Studies have shown that oxidative stress acts as a secondary mechanism that supplements the primary mode of action of some antibiotics [3]. It has been shown that all three major classes of bactericidal antibiotics (aminoglycosides, quinolones and ß-lactams) result in the formation of high doses of reactive oxidative species (ROS), in the form of hydroxyl ions, that significantly increase the killing efficacy and add to the lethality of the drugs [4]. Aerobic bacteria produce trace amounts of superoxide during metabolic pathways using NADH from the TCA cycle to oxidise molecular oxygen to superoxide. Representative antibiotics from each bactericidal antibiotic class have been shown to sequester iron from Iron-Sulphur clusters to promote Fenton mediated hydroxyl radical formation by using the metabolic superoxide [4,5,6,7] (**Figure 2**).

The mechanism of $OH^{\cdot}$ formation can be divided into the following steps:

- Metabolism related NADH depletion to form peroxide through superoxide
- Leaching of Fe^{2+} from intracellular iron sulphur clusters
- Fenton reaction stimulation
- OH radical generation

The hydroxyl radicals cause damage to proteins, DNA and membrane lipids, adding to the killing efficacy of the drugs. The presence of a common oxidative stress and antibiotic killing pathway can potentially help in developing more effective antibiotic therapies.

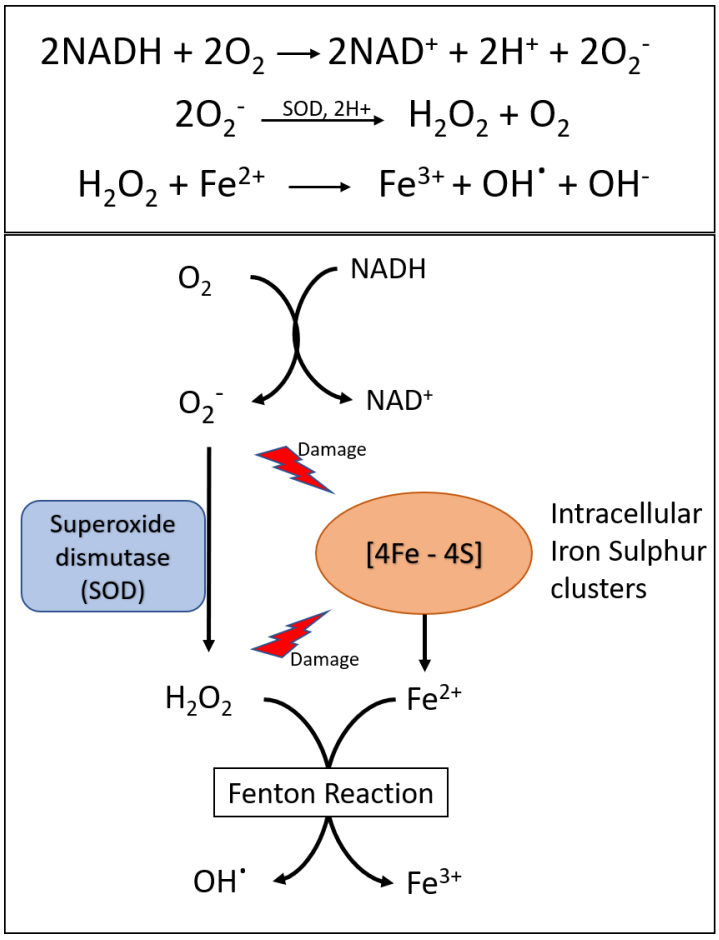

Figure 2: Mechanism of ROS production by Fenton mediated Hydroxyl radical formation

Bacterial oxidative stress response

Cells have inbuilt mechanisms to counteract oxidative stress that spontaneously occurs due to an unwanted transfer of electrons from electron carriers to oxygen as a consequence of aerobic respiration and metabolic processes. ROS formed in the cell cause oxidative stress and result in oxidation of membrane fatty acids leading to lipid peroxidation [8], protein oxidation [9], and also cause DNA damage [10,11]. To counteract these effects of ROS, bacterial cells have many molecular mechanisms to measure ROS and activate antioxidant pathway genes in response to the oxidative stress [12]. However, under oxidative stress where the ROS concentration in the cell is higher than the cell's ability at disposal, the cell viability is compromised. OxyR and SoxRS systems are the most ubiquitously found regulatory systems that use oxidative damage to proteins as physiological cues to trigger global antioxidant responses.

OxyR regulon

OxyR is an H_2O_2 responsive transcriptional regulator that is found across Gram-negative and Gram-positive bacteria [13]. Superoxide generating agents generate sublethal doses of H_2O_2 and induce superoxide resistance which occurs due to the activation of OxyR gene regulated by the *oxyR* regulon [14,15]. The OxyR protein is 34 kDa in size and belongs to the LysR family of transcription factors [13]. The protein responds to H_2O_2 and nitrosothiols [16] though H_2O_2 is the most well characterised activator of OxyR.

In cells, OxyR is found in either its oxidised state (active) or reduced state (inactive) and is post translationally activated [17]. The OxyR polypeptide is found in solution in the tetrameric form [18] and its activation depends on two cysteine residues, Cys 199 and Cys 208, that form a disulphide bond [19] under oxidative stress due to presence of 0.1-1 mM H_2O_2. The oxidised OxyR protein acts as a transcriptional activator for many proteins including glutathione reductase, glutaredoxin, peroxidase and alkyl hydroperoxide-NADPH oxidoreductase, which are all involved in the oxidative stress response. The disulphide bond is re-reduced by glutaredoxin and glutathione which are both part of the oxyR regulon which makes the system autoregulatory during oxidative stress [20] (**Figure 3**).

Although OxyR mostly acts as a transcriptional activator, there are some examples where OxyR acts as a repressor [21,22]. It should be noted that OxyR can function as an activator and repressor for a gene, depending on its oxidation states [23]. In *E. coli,* OxyR binds as a tetramer to an approximately 40 bp-region containing two tandem ATAG-N7-CTAT repeats at about 10 bp intervals, located just upstream of the -35 box of at least 30 regulon members in *E. coli* [24,27]. Though the intramolecular cysteine disulfides have been shown to be required for the activity of OxyR[34], it still remains to be understood if this cysteine disulfide formation is required for oligomerization and DNA binding.

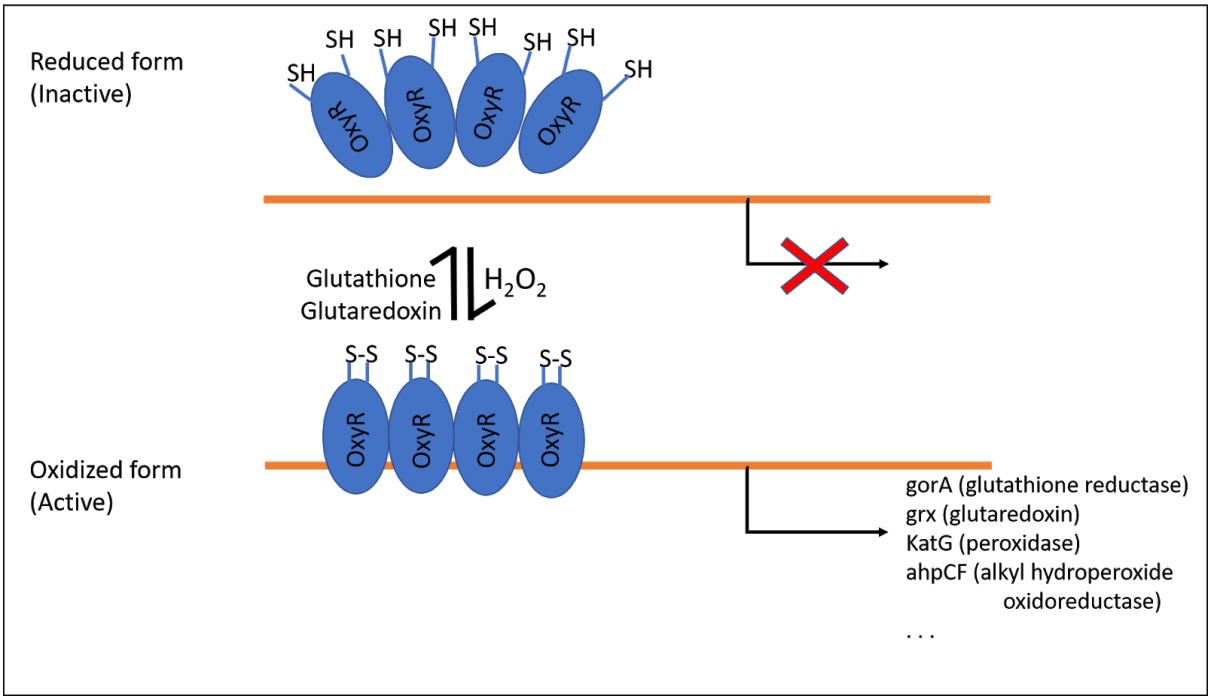

Figure 3: Cartoon depiction of OxyR regulation

This work standardizes the experimental conditions required to study (i) the role of cysteine disulfides in DNA binding and activity of OxyR and (ii) the involvement of OxyR in conferring resistance against bactericidal antibiotics.

Materials and methods

Strains used

DH5α: high transformation efficiency *E. coli* strain used for plasmid amplification
Rosetta star: enhanced protein expression *E. coli* strain used for protein expression
NA1000: Wild type *Caulobacter crescentus*
SKR2284: ΔOxyR1; deletion mutation 1 of OxyR in a WT *Caulobacter* background
SKR2285: ΔOxyR2; deletion mutation 2 of OxyR in a WT *Caulobacter* background
SKR2161: D4; gain of function mutation in *Caulobacter* OxyR in a WT *Caulobacter*
SKR2162: D26; gain of function mutation in *Caulobacter* OxyR in a WT *Caulobacter*
SKR2163: D32; gain of function mutation in *Caulobacter* OxyR in a WT *Caulobacter*

The gain of function mutations were isolated in lab by screening for colonies capable of growth in the presence of a reducing agent. The mutants which mapped to OxyR were used for the study here.

Plasmid transformation

Competent cells (DH5α for plasmid amplification and Rosetta star for expression and purification) from -80°C were thawed on ice (for 10-15 mins) and 1µL of plasmid was added to 50µL of competent cells and flicked to mix while on ice. The mixture was incubated on ice for 25 minutes and given a heat shock at 42°C using a water bath for 2 minutes followed by incubation in ice again for 5 minutes. The cells were removed from ice and 900µL of LB broth was added and incubated at 37°C while shaking for 1 hour to recover them. 110µL of culture was asymmetrically plated on the selective antibiotic LB agar plate and incubated overnight at 37°C.

Plasmid isolation

Cultures of transformed DH5α were grown overnight from a single colony at 37°C in the selective antibiotic LB media. 3mL cells were pelleted by centrifugation at 9000 RPM for 2 mins and supernatant (growth media) was discarded. The pellet was resuspended in 250µL Resuspension buffer, 250µL Lysis buffer was added and inverted to mix till viscous. 350µL of Neutralisation buffer was added and inverted to mix till precipitate forms. The sample was centrifuged at 13,200 RPM for 10 mins to separate precipitate. The supernatant was added to vacuum tubes and washed twice with 600µL Wash (A4) buffer. Vacuum drained tubes were centrifuged at 11,000 RPM for 2 mins to remove residual ethanol from A4 buffer. Vacuum tubes were kept in labelled 1.5mL microcentrifuge tubes and 30µL warmed Elution buffer was added and incubated at room temperature for 15 mins. The tubes were centrifuged at 10,000 RPM for 1.5 mins and the eluted plasmid was stored at -20°C.

Induction and cell pellet storage

Single colony of Rosetta star transformed with pSKR 299 (pET-28a with Caulobacter OxyR insert) was inoculated from a freshly transformed plate (no more than 3 days old) and grown overnight at 37°C. The culture was diluted 1:20 the next day and allowed to grow till it reached O.D. of 0.2 at 37°C when it was shifted to 29°C till the culture reached an O.D. of

0.4. the culture was induced with IPTG such that final concentration was 1mM and allowed to grow for 6 hours at 29°C. The culture was centrifuged at 8,000 RPM for 10 minutes at 4°C, resuspended in 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4) to wash off remaining media and centrifuged again at 8,000 RPM for 10 minutes at 4°C, supernatant was discarded and pelleted cells stores at -80°C till further processing.

Sonication

For minor culture

The pellet from 8 mL of culture was resuspended in 1mL chilled OxyR lysis buffer (50mM NaH_2PO_4, 300mM NaCl, 10mM Imidazole, 1X protease inhibitor). The sample was chilled on ice while the sonication probe was cleaned with 70% ethanol. The sample was sonicated at 70% amplitude with 30s on and 30s off pulses for 10 cycles or 5 mins while on ice to prevent heating up. The sample was sonicated more if needed till it turned clear. The chilled sonicated sample was centrifuged at 10,000 RPM for 10 mins to pelletise the cell debris. The supernatant is transferred to a new 1.5mL microcentrifuge tube and stored at -20°C if not used immediately.

For major culture

The pellet from 500-1000 mL of culture was resuspended in 25mL chilled OxyR lysis buffer I (used for Ni NTA without FPLC) (50mM NaH_2PO_4, 300mM NaCl, 10mM Imidazole) or chilled OxyR lysis buffer II (20mM Tris HCl pH8, 150mM/300mM NaCl). The sample was chilled on ice while the sonication probe was cleaned with 70% ethanol. The sample was sonicated at 70% amplitude with 3s on and 3s off pulses for 5 mins while on ice to prevent heating up. The sample was sonicated more if needed till it turned clear. The chilled sonicated sample was centrifuged at 10,000 RPM for 10 mins to pelletise the cell debris. The supernatant is transferred to a new 50mL centrifuge tube and stored on ice for not more than 20 minutes before purification.

Expression check using SDS-PAGE

Gel casting

The glass plates were assembled in the BioRad gel casting apparatus. The components for the resolving gel (12.5% acrylamide, Tris 0.5M-pH 8.8) were mixed and poured into the glass plates with 2cm on top. A layer of isopropanol was added over the top of the resolving gel to prevent meniscus and bubble formation in the resolving gel. The resolving gel was allowed to stand at room temperature till the gel set.

Once the gel solidified, isopropanol was drained and blotted from between the glass plates. The stocking gel (6% acrylamide, Tris 1.5M-pH 6.8) was mixed and poured on top of running gel so that the plates are completely full and the comb was immediately placed making sure no air bubbles were incorporated. The gel was left to stand at room temperature to set.

Sample preparation

From the cell lysate supernatant, 30µL was taken and 10µL of 4X SDS loading dye (500 mM Tris-HCl pH 6.8, 10% SDS, 5% glycerol, 500mM β-mercaptoethanol, 50 mM EDTA, 0.08 % bromophenol blue) was added to it and vortexed to mix. The pellet was resuspended in 30µL of 1X SDS loading dye was added and vortexed to mix. The tubes were incubated at 99°C for

12 mins. Samples were given a 7 second short spin to collect all the condensation back in the sample pool.

Sample loading

The comb was removed from the gel casting glass plates and the plate was assembled into the gel running apparatus. 1X SDS running buffer was added to both chambers of the apparatus. The prepared samples are added to the gel wells. The gel was run at 100V till the dye front passes from the bottom of the gel.

Coomassie staining

The gel was removed from the apparatus and the glass plates carefully without breaking while holding under a gentle stream of water. 50 mL Coomassie staining solution (50% ethanol, 10% Glacial acetic acid, 0.25%Coomassie brilliant blue) was added and brought to a boil and gently shaken for 30 mins. The stain is poured off and stored again for reuse and the gel is rinsed with water to wash off the residual stain. 50mL de-staining solution (20% ethanol, 10% glacial Acetic acid) was added to the stained gel, brought to a boil and shaken gently for two hours till the gel is visibly de-stained. The de-staining solution is poured out and stored for reuse and the gel was rinsed with water again. The gel was imaged in the gel doc.

Western blot

PVDF Transfer

A piece of the PVDF membrane was measured, cut and dipped in methanol. A just run PAGE gel was removed from the glass plates and transferred into MilliQ water. The PVDF membrane was transferred from methanol into fresh 1X transfer buffer (25 mM Tris HCl pH 8.3, 192 mM glycine, 10%methanol). The transfer cassette was assembled with the gel towards the negative plate and the PVDF membrane towards the positive plate without air bubbles. The cassette was placed in the transfer apparatus and run at 90V for 3 hours at 4°C for heat dissipation. The membrane was removed from the transfer assembly cassette and dipped in methanol gain for 5 mins and dried before storage.

Blot development

The blot (transferred PVDF membrane) was dipped in methanol for 5 mins and then washed with 1X TBS (20 mM Tris HCl pH 7.6, 150 mM NaCl) for 5 mins while shaking gently. The blot was blocked using a solution containing 5% milk and 1% TWEEN. The blot was then stained with rabbit derived primary antibody against OxyR in a 5% milk solution (1:10,000) with mild shaking for 1 hour at room temperature. The blot was then given three washes with 1X TBS for 5 mins each while shaking gently. Secondary antibody, donkey anti-rabbit, was then added to a 5% milk solution (1:5,000) and added to the blot and kept to shake gently for an hour. The blot was washed after this four times with 1X TBS for 5 mins each before transferring into fresh 1X TBS for imaging.

Blot imaging

The blot is imaged immediately after development within 10-20 minutes and kept in 1X TBS till imaging. ECL western blot substrate is used to visualise the bands on the PVDF membrane. The luminol (solution A) and peroxide (solution B) are mixed in 1:1 ratio (500µL

each) to activate the substrate and kept away from light. The blot is saturated in the substrate for 5 minutes in low light conditions and wrapped in clear plastic film to prevent drying for taking an image using iBright imaging system in the chemiblots setting for the autoexposure suggested time, and half the time in case saturation is very high.

Non-reducing PAGE

Gel casting

The glass plates were assembled in the BioRad gel casting apparatus. The components for the resolving gel (12.5% acrylamide, Tris 0.5M-pH 8.8) were mixed and poured into the glass plates with 2cm on top. A layer of isopropanol was added over the top of the resolving gel to prevent meniscus and bubble formation in the resolving gel. The resolving gel was allowed to stand at room temperature till the gel set.
Once the gel solidified, isopropanol was drained and blotted from between the glass plates. The stacking gel (6% acrylamide, Tris 1.5M-pH 6.8) was mixed and poured on top of running gel so that the plates are completely full and the comb was immediately placed making sure no air bubbles were incorporated. The gel was left to stand at room temperature to set.

Sample preparation

From the cell lysate supernatant, 30µL was taken and 10µL of 4X SDS loading dye (500 mM Tris-HCl pH 6.8, 10% SDS, 5% glycerol, 500mM β-mercaptoethanol, 50 mM EDTA, 0.08 % bromophenol blue) was added to it and vortexed to mix. The pellet was resuspended in 30µL of 1X SDS loading dye and vortexed to mix. The tubes were incubated at 99°C for 12 mins. Samples were given a 7 second short spin to collect all the condensation back in the sample pool.

Sample loading

The comb was removed from the gel casting glass plates and the plate was assembled into the gel running apparatus. A 1X TBE running buffer was added to both chambers of the apparatus. The prepared samples are added to the gel wells. The gel was run at 100V till the dye front passes from the bottom of the gel.

Ni-NTA Purification (not to be followed by gel filtration/FPLC)

The supernatant from the sonicated cell lysate was used for purification of OxyR product and maintained at 4°C at all times. The column apparatus was washed with MilliQ water and was set up and secured upright on a column stand. The column was packed with 3 mL of Ni-NTA agarose (1 mL in case of minor culture sample) without drying it out. The beads were first washed with 50mL OxyR Lysis buffer I (50mM NaH_2PO_4, 300mM NaCl, 10mM Imidazole). The cell lysate supernatant was then passed through the column. The column was washed off the unbound proteins using 50mL of the wash buffer (50mM NaH_2PO_4, 300mM NaCl, 20mM Imidazole) followed by an extra wash with 50 mL of wash buffer with 50mM Imidazole (50mM NaH_2PO_4, 300mM NaCl, 50mM Imidazole). The protein was eluted from the column using 25 mL of elution buffer (50mM NaH_2PO_4, 300mM NaCl, 250mM Imidazole). The passthrough was collected in 1.5mL microcentrifuge tubes numbered sequentially. 20µL from each of the collection tubes was run on an SDS PAGE to check for the sample(s) with the

highest concentration. Samples with high concentrations of protein were stored at -80°C after adding glycerine (20% final concentration).

Dialysis

One tube of purified protein of a high concentration was thawed on ice. The sample was added to the dialysis membrane and suspended in 1L chilled dialysis buffer (20mM Tris-HCl; ph7.5, 50mM NaCl, 2.5mM MgCl2, 0.45mM EDTA, 0.5mM DTT, 0.05% Nonidet P-40, 10% Glycerol) and stirred for 1 hour at 4°C. The buffer was replaced after one hour with fresh chilled dialysis buffer and stirred for two more hours. The protein was kept chilled on ice and concentrated using a concentrating tube at 4°C. The concentrated protein was aliquoted into 30µL volumes and stored at -80°C.

Bradford assay

A working stock solution of 1mg/ml BSA was prepared from a 10mg/ml stock solution made and stored at -20°C. Standard solutions were made according to the table below (**Table 1**) and used to construct the Bradford standard graph. 4µL of each standard solution was taken in a 96 well plate. Three dilutions of the protein sample were made; 1/5X,1/10X and 1/20X by adding 1µL of protein sample to 4µL, 9µL and 19µL of the buffer solution the protein was currently dissolved in. 4µL of each of these sample dilutions were also added to the 96 well plate. 200µL of Bradford reagent was added to each of the 10 standard concentration wells and the three sample protein concentration wells. Absorbance was measured at 595nm and a standard graph was plotted using the BSA solutions and the protein concentration was estimated using the BSA standard.

S. No	Concentration	Volume of working stock	Volume of MilliQ
1	0.1	10µL	90µL
2	0.2	20µL	80µL
3	0.3	3µL	70µL
4	0.4	40µL	60µL
5	0.5	50µL	50µL
6	0.6	60µL	40µL
7	0.7	70µL	30µL
8	0.8	80µL	20µL
9	0.9	90µL	10µL
10	1.0	100µL	0µL

Table 1: Bradford standard solution ratios

Ni-NTA Purification (to be followed by gel filtration/FPLC)

The supernatant from the sonicated cell lysate was used for purification of OxyR product and maintained at 4°C at all times. The column apparatus was washed with MilliQ water and was set up and secured upright on a column stand. The column was packed with 3 mL of Ni-NTA agarose without drying it out. The beads were first washed with 50mL OxyR Lysis buffer I (150mM/300mM NaCl, 20mM Tris HCl pH8). The cell lysate supernatant was then passed through the column. The column was washed off the unbound proteins using 50mL of the

wash buffer (150mM/300mM NaCl, 20mM Tris HCl pH8, 20mM Imidazole) followed by an extra wash with 50 mL of wash buffer with 50mM Imidazole (150mM/300mM NaCl, 20mM Tris HCl pH8, 50mM Imidazole). The protein was eluted from the column using 25 mL of elution buffer (150mM/300mM NaCl, 20mM Tris HCl pH8, 150mM Imidazole). The passthrough was collected in 1.5mL microcentrifuge tubes numbered sequentially in 1mL increments. 10µL from each of the collection tubes was run on an SDS PAGE to check for the sample(s) with the highest concentration. The tubes with the highest concentrations were pooled together for dialysis or concentration as needed.

Dialysis

All tubes with high enough concentration of protein from the Ni-NTA elution were pooled into one sample. The sample was added to the dialysis membrane and suspended in 1L chilled dialysis buffer (20mM Tris-HCl; ph7.5, 200mM NaCl) and stirred for 1 hour at 4°C. The buffer was replaced after one hour with fresh chilled dialysis buffer and stirred for two more hours.

Protein concentration

A 6mL concentration tube (vivo-spin 6) was washed with 5mL MilliQ water and 5mL of 1X Lysis buffer. The tubes with the highest concentration of Ni NTA column elute were pooled together into a single sample or the dialysed protein sample was used. The sample was loaded into the concentration tube and centrifuged till a required concentration or volume was reached or till a precipitate was formed. The resultant concentrated product was used to load into the gel filtration column.

Gel filtration purification

The concentrated sample (2ml final volume) was loaded into the loading tube in the gel filtration apparatus. A Hiload 16/60 Superdex 200 column prep grade column was used for the purification step and the elution fractions with the highest concentrations of the expected size range were run on an SDS PAGE gel to check purity and pooled together and concentrated. The concentrated purified protein product was stored as 10µL aliquots at -80°C.

Nuclease activity

10µL of plasmid DNA was added to two 0.6 mL microcentrifuge tubes each. 5µL of concentrated purified protein was added to one tube and 5µL dialysis buffer was added to the other one. The volume of both the mixtures was made 30µL using sterile MilliQ water. The mixtures were incubated at 37°C for 2 hours and 12 hours. 5µL of 6X purple loading dye was added to each sample and mixed thoroughly. The samples were loaded into an 8% agarose gel with EtBr and run at 100V till the dye front runs out of the gel. The gel was visualised in ultraviolet light in the Gel doc.

Construction and Annealing of oligomers

15 identified potential targets of OxyR were chosen from the Caulobacter full genome and 500bp long sequences from upstream of their start sites were used. These sequences were aligned using SnapGene software to identify within these, five sequences of high consensus and these were used for making alignment consensus sequences. The consensus sequence

was aligned to the 500 bp long sequences upstream of the start codons of two most well studied proteins known to be transcriptionally activated by OxyR (KatG and Peroxiredoxin). Oligomers were constructed around the potential binding sites found in these sequences and both forward and backward sequences were individually ordered. Once received, each of the constructs were dissolved in sterile MilliQ water as directed on the MSDS sheet. 10µL of equimolar forward and backward oligomers of all 6 sets were added to respective PCR tubes along with 10µL 10X annealing buffer (100mM Tris HCl pH 7.5, 0.5M NaCl, 10mM EDTA) and volume made to 100µL using sterile MilliQ. The PCR tubes were heated to 95°C for 10 minutes and cooled down at a rate of 1°C/minute till they were at 4°C to slowly anneal the forward and backward oligomers. The tubes were then maintained at 4°C to avoid remelting before storage at -20°C till use.

EMSA (Electrophoretic Mobility Shift Assay)

Different concentrations of purified OxyR protein products (purified using Ni-NTA column and FPLC purified) were diluted in binding buffer (20mM Tris HCl pH 7.5, 50mM NaCl, 2.5mM $MgCl_2$, 0.45mM EDTA, 10% Glycerol) to usable concentrations. 40ng or 80ng of annealed DNA oligomers were added to these using binding buffer as the solvent. The mixtures were incubated at 25°C for 30 minutes to allow binding of OxyR to oligomeric constructs. The samples were then loaded onto a 6% non-reducing gel and run at 4°C till the dye front had run about 3/4th the way. The gels were carefully stained using an EtBr bath (5µL in 50mL MilliQ) and imaged under UV light to visualise the bands of protein bound and unbound DNA.

Antibiotic susceptibility test

All 6 strains of *Caulobacter crescentus* (NA1000, ΔOxyR1, ΔOxyR2, D4, D26 and D32) were grown overnight at 29°C to get a good cell count. The cultures were diluted the next day to an O.D. less than 0.2 and were allowed to grow at 29°C to an O.D. 0.4-0.6. they were then diluted to O.D. 0.3 using sterile MilliQ. Each of these were again diluted 1:10 in sterile MilliQ 4 times serially such that if O.D. 0.3 is considered 10^0 (or 1), we have the dilutions 10^0, 10^1, 10^{-2}, 10^{-3} and 10^{-4} in each of the 6 strains. 10µL of each of the dilutions O.D. each strain are spotted onto PYE agar plates in the presence and absence of sublethal doses of antibiotics (0.25µg/ml Gentamicin, 1µg/ml Kanamycin, 5µg/ml Spectinomycin, 2.5µg/ml Ciprofloxacin, 0.5µg/ml Gentamicin, 2.5µg/ml Kanamycin, 15µg/ml Spectinomycin, 5µg/ml Ciprofloxacin). The plates were incubated for 24 hours at 29°C and imaged.

Determination of minimal inhibitory concentration (MIC)

For finding the MIC of the 4 antibiotics, 5 or more concentrations of each of the antibiotics were used in liquid media (PYE broth). All 6 strains of *Caulobacter crescentus* (NA1000, ΔOxyR1, ΔOxyR2, D4, D26 and D32) were grown for 24 hours at 29°C in 96 well plates in the presence of Gentamicin (0µg/ml, 0.05µg/ml, 0.1µg/ml, 0.25µg/ml, 0.5µg/ml), Kanamycin (0µg/ml, 0.1µg/ml, 0.25µg/ml, 0.5µg/ml, 1µg/ml), Spectinomycin (0µg/ml, 0.5µg/ml, 1µg/ml, 2.5µg/ml, 5µg/ml) and Ciprofloxacin (0µg/ml, 1µg/ml, 2µg/ml, 4µg/ml, 6µg/ml, 8µg/ml, 10µg/ml, 20µg/ml, 30µg/ml, 40µg/ml). O.D. 600 was measured at 24 hours to select a antibiotic concentration at which WT grows half as well in the presence of antibiotic as in the absence of antibiotic. Growth curves were done for the strains with antibiotic concentrations ascertained this way.

Growth in liquid media

All 6 strains of *Caulobacter crescentus* (NA1000, ΔOxyR1, ΔOxyR2, D4, D26 and D32) were grown overnight at 29°C to get a dense primary inoculum. Same number of cells were calculated and added to 5mL cultures without antibiotic, 0.1µg/mL Gentamicin, 0.25µg/mL Kanamycin, 10µg/mL Spectinomycin and 1µg/mL ciprofloxacin such that the initial O.D. of all cultures is 0.05. 200µL of culture was taken every hour and measured for O.D. 600 and plotted.

Results

Expression of recombinant OxyR in *E. coli*

To overexpress the recombinant *Caulobacter* OxyR in *E. coli*, pET28a plasmid expressing His6-OxyR from an IPTG inducible T7lac promoter was transformed into *E. coli* Rosetta(DE3)pLysS expression system (Novagen).

To confirm that OxyR was obtainable in the soluble fraction of the cell lysate, 8ml of culture of OD 0.4 was induced for 6 hours before palletisation and resuspended in lysis buffer (50mM NaH_2PO_4, 300mM NaCl, 10mM Imidazole) before sonication (amplitude 75%, pulse 30-30, time 5 mins). The cell lysate was centrifuged to separate the soluble and non-soluble components and both fractions were run on an SDS-PAGE to determine the nature of OxyR. Upon Coomassie staining, an appropriate band corresponding to the size of monomeric His6-OxyR (~34kDa) was observed to be overexpressed in the soluble fraction **Fig4A**.

To check whether the band observed in the SDS-PAGE was OxyR, immunoblot using rabbit-derived polyclonal antibodies of OxyR was performed on the soluble fraction. A strong signal was detected, as seen in **Fig4B**, corresponding to the band seen in the Coomassie stained gel confirming the presence of OxyR in the soluble fraction post sonication. To check for expression, immunoblots were done using the soluble fractions from cells grown in the presence and absence of IPTG. The OxyR expression was seen to be enhanced greatly in the induced sample, **Fig4C**.

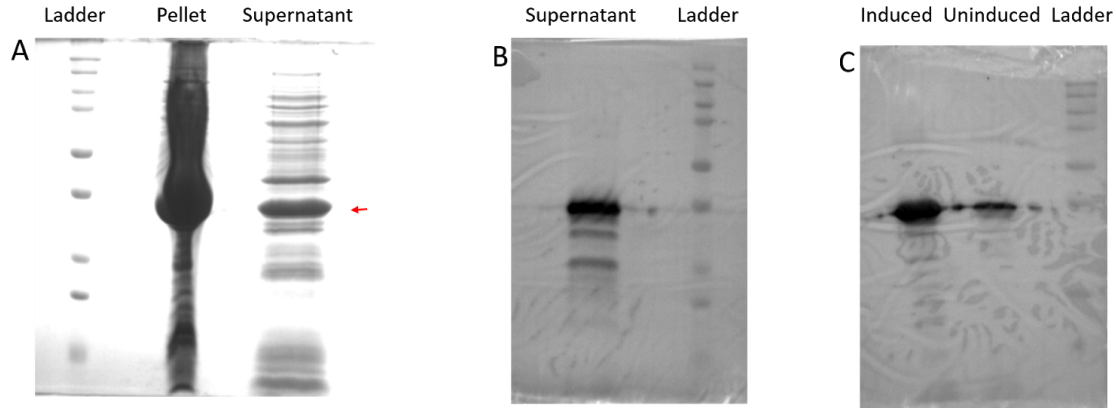

Figure 4: A. Coomassie stained SDS PAGE. Red arrow marks the 34kDa band in soluble fraction (right lane). **B.** Western blot of soluble fraction of induced cell lysate showing a prominent OxyR band. **C.** differential expression of OxyR in induced vs uninduced cell lysate soluble fraction seen in western blot.

OxyR purification using Ni-NTA and dialysis

To test the efficacy of purification, an 8mL culture induced with IPTG for 6 hours and was pelletised for purification of OxyR protein. The pellet was resuspended in lysis buffer, sonicated and the soluble fraction was passed through a Ni-NTA agarose column (see Methods for details). The different collection tube samples were run on an SDS PAGE (**Fig5A**) and the tubes with high protein concentrations (1,2,3) were stored in 20% glycerol.

To corroborate the bands seen in the purified samples, the purified product was run alongside uninduced and induced cell lysate soluble fractions. This showed multiple bands (**Fig5B**) that did not coincide with 34kDa size that were likely oligomeric and degradation products and other impurities.

A 2-litre culture was induced with IPTG at OD 0.4 and grown for 6hrs at 29°C and pelletized. The pellet was resuspended in lysis buffer and sonicated to lyse the cells and the soluble OxyR was purified using a Ni-NTA agarose column. To refine purification further, an extra buffer wash was given with 50mM Imidazole concentration. The collection tube samples were run on an SDS PAGE (**Fig5C**) and the tubes with high protein concentrations (2,3,4,5) were stored in 20% glycerol.

Half of the volume collected in tube 2 of the major culture was dialysed in dialysis buffer for 3 hours and concentrated from 0.089 ng/µL to 2.42ng/µL by centrifuging the dialysed sample in a concentrating tube. The resulting concentrated protein was stored as 30µL aliquots at -80°C.

Since the purified protein product still showed multiple bands, to see whether purification was efficient or was a mix of proteins, a western blot of purified product was seen, **Fig 5D.** The blot showed multiple bands of smaller intensity of various sizes both smaller and larger than 34kDa size.

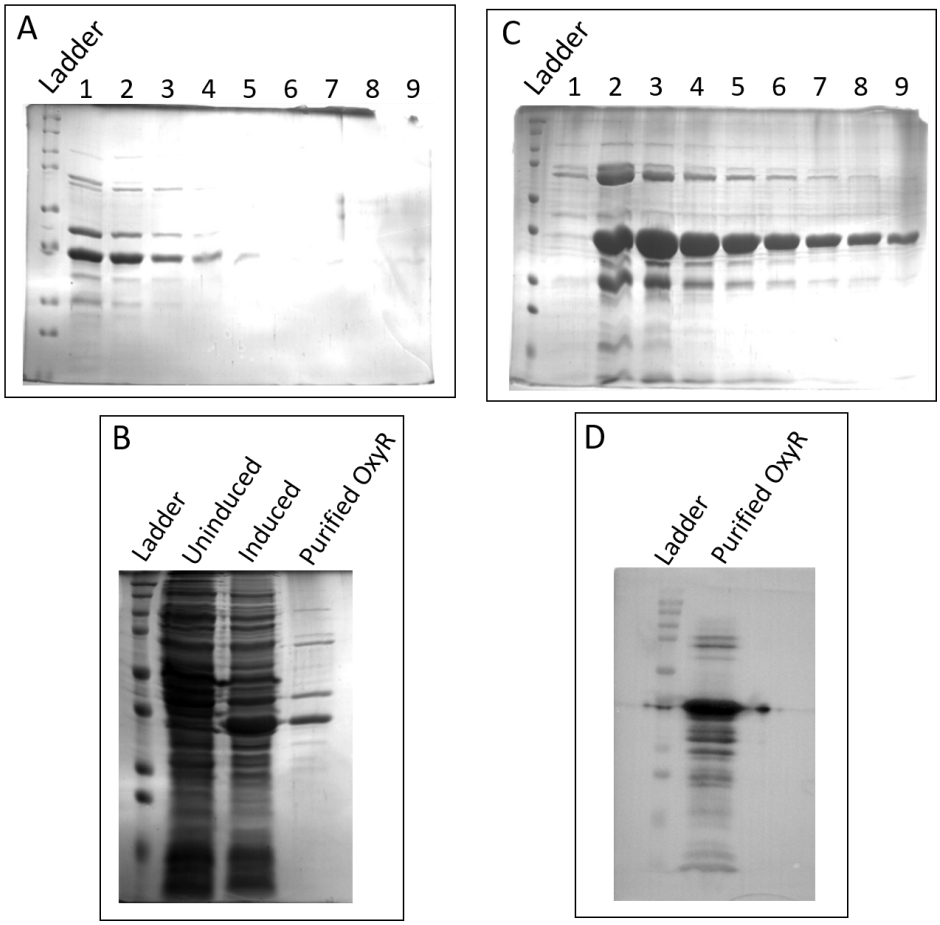

Figure 5: A. Coomassie stained SDS PAGE of minor culture collection tubes. **B**. Coomassie stained SDS PAGE of uninduced lysate, induced lysate and purified product. **C.** Coomassie stained SDS PAGE of major culture collection tubes. **D.** Western blot of OxyR purified product.

OxyR purification using Ni-NTA and Gel Filtration

Since the protein product after Ni-NTA purification still had considerable impurities, a second purification step was added. The Ni-NTA purification collection fractions (2,3,4,5,6) (**Fig 6A**) were pooled together, dialysed (20mM Tris-HCl; pH7.5, 50mM NaCl, 2.5mM MgCl2, 0.45mM EDTA, 0.5mM DTT, 0.05% Nonidet P-40, 10% Glycerol) and concentrated to reduce the volume to 3.2mL. The apparatus used for running the gel filtration (FPLC) column was at room temperature (25°C). When he collected fractions were concentrated (500µL of 1.8mg/mL) and run on an SDS PAGE gel and stained with Coomassie stain, showed more bands of impurity after the second purification step as compared to after the Ni-NTA purification (**Fig 6B**). To ascertain whether the bands seen on the Coomassie stained were impurities or OxyR oligomers and degradation products thereof, two sets of the purified protein and ladder were run on a single SDS PAGE gel and one set from half the gel was stained with Coomassie (**Fig 6C**) and the other set was used for western blotting (**Fig 6D**) for comparative analysis. The multiple bands seen in the western blot other than monomeric OxyR were most likely impurities, oligomers and their degradation products. This was expected to be because of the second purification step being conducted at room temperature. The temperature fluctuations might likely have caused protein oligomerisation or degradation to occur. The possibility that the buffer system being used was incompatible with the protein was also considered.

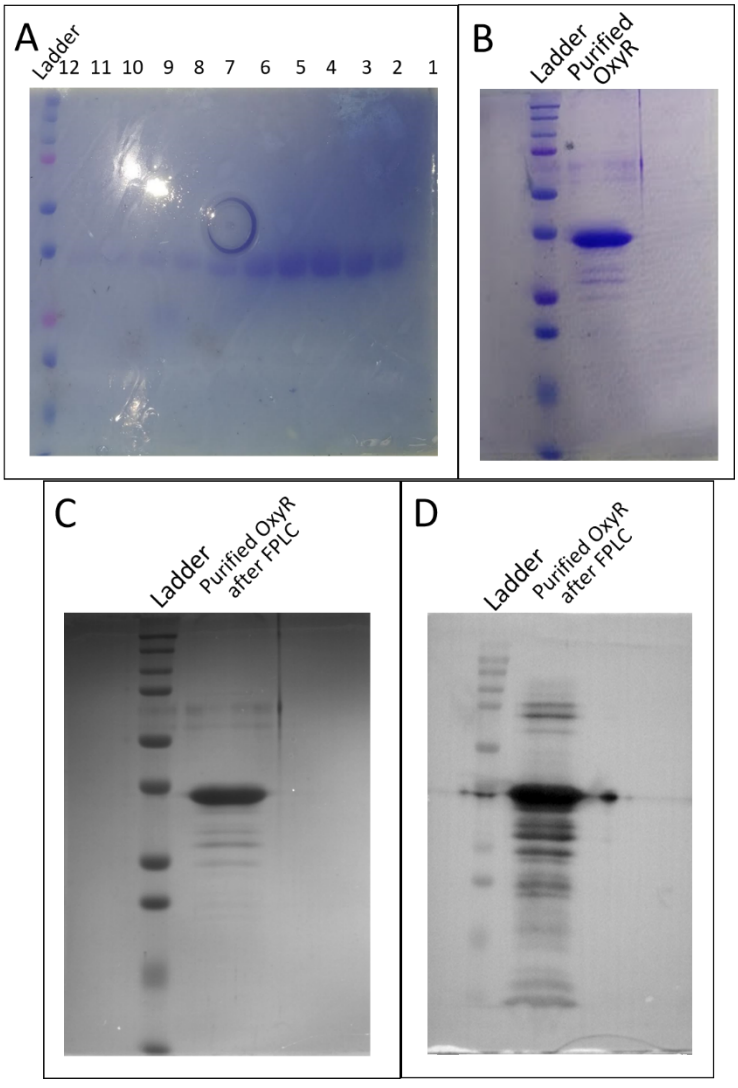

Figure6: A. Coomassie stained SDS PAGE gel for the collection fractions of Ni-NTA purification. B. Coomassie stained SDS PAGE gel for the combined, concentrated protein product after gel filtration. C. Half gel used for Coomassie stained SDS PAGE gel of the gel filtration product. D. western blot from half gel of the gel filtration product.

OxyR purification using Ni-NTA and Gel Filtration at 4°C

To reduce the non-specific bands on the gel, two things were changed and the experiment was repeated. The buffer system was changed (150mM NaCl, 20mM Tris HCl pH8), and to ensure the temperature shock wasn't responsible for protein degradation, the experiment was conducted in a cold room (maintained at 4°C). The Ni-NTA collection fractions (2,3,4,5) (**Fig7A**) were concentrated and loaded into the cold FPLC column. The dialysis step was skipped as the buffer systems used for Ni-NTA purification and gel filtration were very similar in both composition and concentration. Elution fractions (**Fig7B**) showed really low yield even after concentration (100µL of final concentration 0.8mg/mL) as a lot of protein precipitated out during the concentration step. To prevent that, in the next iteration, the buffer system was modified again (gel images not included) to increase salt concentration (300mM NaCl, 20mM Tris HCl pH8) and a final concentration of 23mg/mL was obtained, though it could potentially have been concentrated further it was not due to insufficient volume.

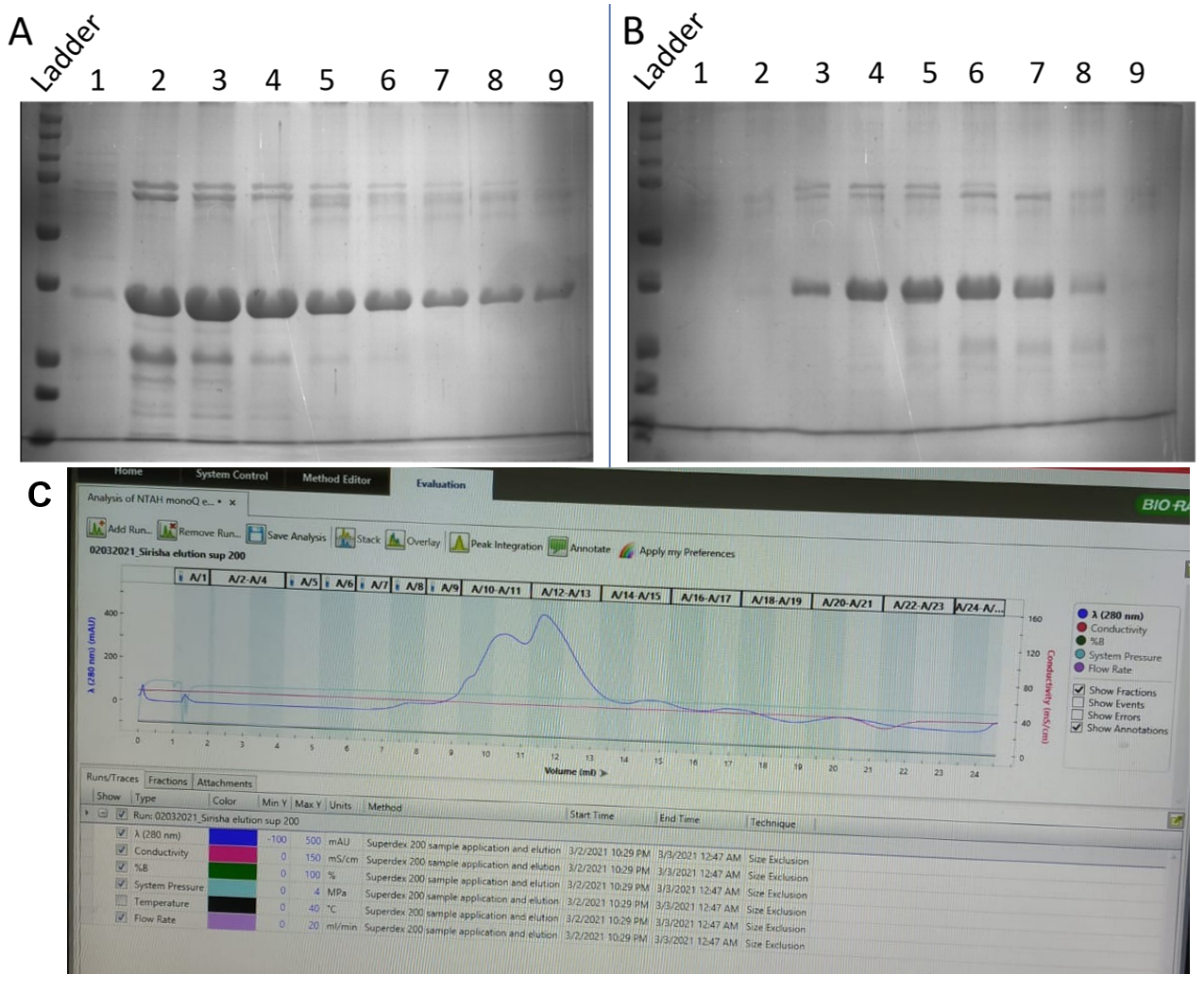

Figure 7: **A**. Coomassie stained SDS PAGE of Ni NTA collection tubes. **B**. Coomassie stained SDS PAGE of FPLC collection tubes. **C**. Elution profile of gel filtration column.

Native gel of FPLC purified OxyR

Since SDS PAGE is highly reducing and the sample is boiled before loading, to check whether the processing of the sample causes degradation of the protein, the purified protein product was run on a native gel in the presence of SDS and BME, just BME and without either (**Figure 8B**). Since the protein did not run very far in the gel, it can be assumed that boiling and addition of the reducing agent are at least partly responsible for the multiple nonspecific bands seen even after multiple purifications though, most likely, the protein is still not in a completely pure form (single state).

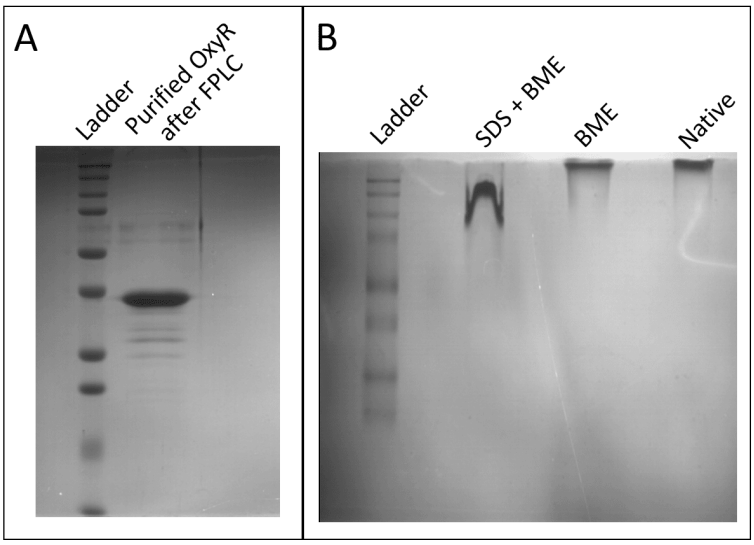

Figure 8 A. FPLC purified protein sample run on an SDS PAGE and Coomassie stained. **B.** FPLC purified protein sample processed with SDS and BME, BME and without either run on a native gel and Coomassie stained.

Nuclease activity of Purified OxyR

Before analysing the DNA binding ability of the purified OxyR using EMSA , it needed to be ascertained that the purified protein product does not have any nuclease activity. For this, plasmid containing the WT oxyR construct (pSKR299) was incubated at 37°C hours 2 hours **Fig 9A**, and 12 hours **Fig 9B**, in the presence and absence of protein. In both cases, no degradation of the plasmid was observed.

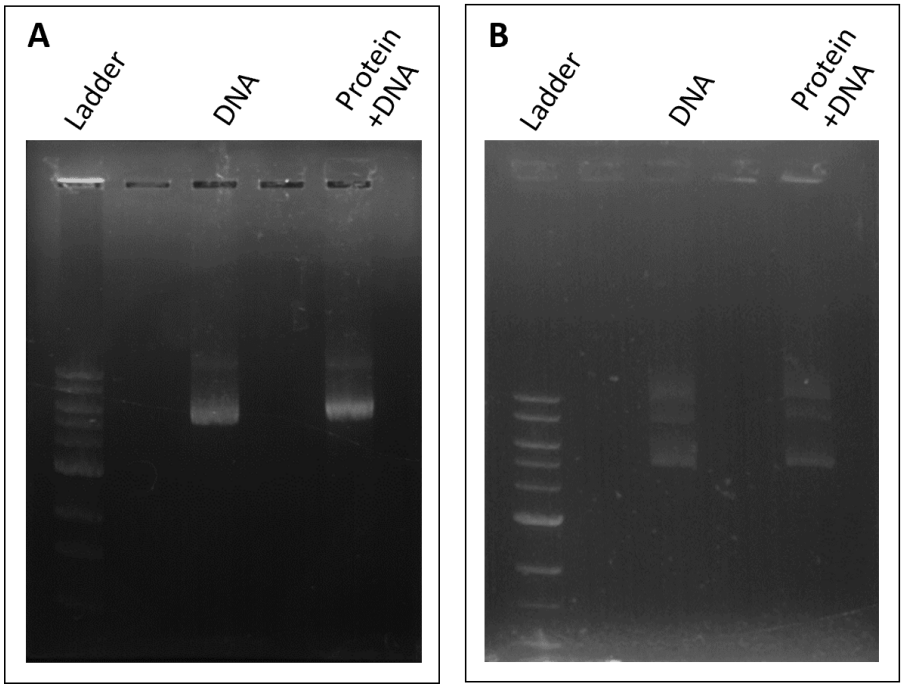

Figure 9 A. Nuclease activity after 2 hours of incubation with purified protein (left lane) and without purified protein (right lane). **B.** Nuclease activity after 12 hours of incubation with purified protein (right lane) and without purified protein (left lane).

Oligomer construction for EMSA assay

From the complete *Caulobacter* full genome, 500 base long regions upstream of the start codon were taken from 15 different proteins that had been previously shown transcriptional activation by OxyR [35]. A basic alignment was done using these sequences using Snapgene software (**Figure10**). Using the obtained alignment and the binding motif described by Silva et al. [26], motifs upstream of OxyR-regulated *katG* gene and peroxiredoxin were chosen for further studies using EMSA. . Synthetic oligomers were constructed for each gene as shown in **Table 2**.

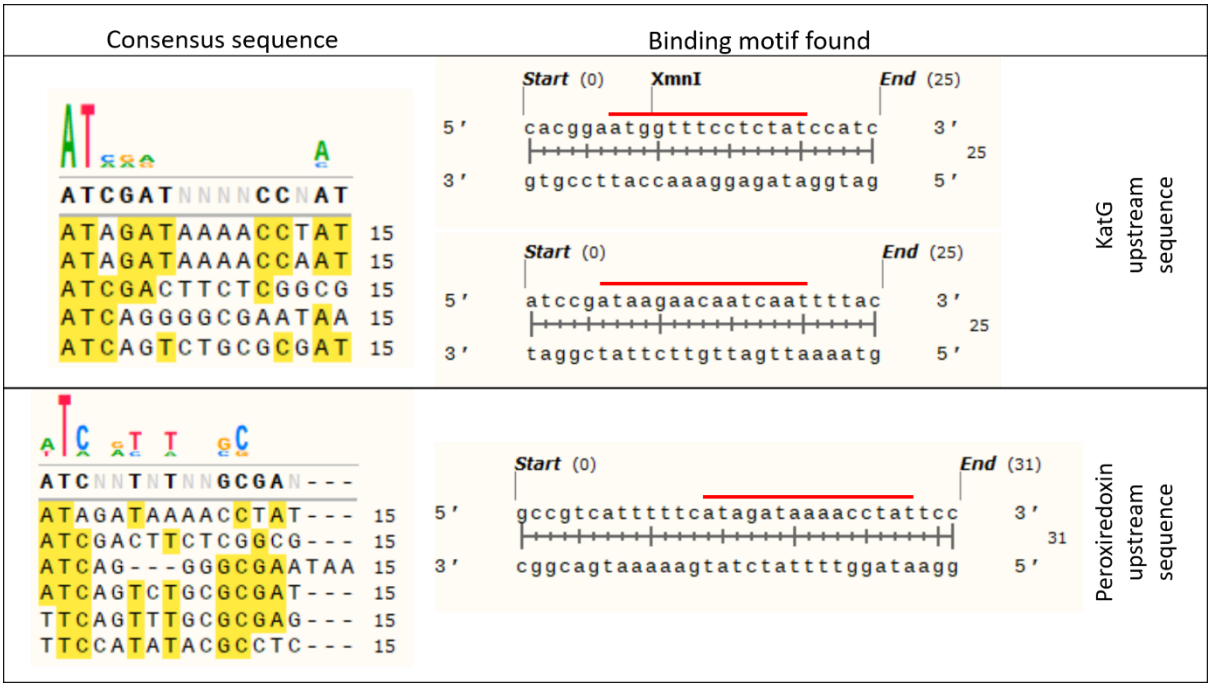

Figure 10: Consensus sequence obtained from alignments and binding motifs found in KatG upstream sequence and peroxiredoxin upstream sequence using consensus sequences. The exact binding motif as verified from Silva. et. al. is marked by the red bars in each case.

S.No	Construct	+/-	Sequence 5' - 3'	GC content
1	katG construct1 25bp	Positive	cacggaatggtttcctctatccatc	48%
2	katG construct1 25bp	Negative	gatggatagaggaaaccattccgtg	48%
3	katG construct1 50bp	Positive	cacggaatggtttcctctatccatccgataagaacaatcaattttaccaa	38%
4	katG construct1 50bp	Negative	ttggtaaaattgattgttcttatcggatggatagaggaaaccattccgtg	38%
5	katG construct2 28bp	Positive	ccatccgataagaacaatcaattttacc	36%
6	katG construct2 28bp	Negative	ggtaaaattgattgttcttatcggatgg	36%
7	katG construct2 50bp	Positive	atccgataagaacaatcaattttaccaatggcgaaaaagagcggcttcta	38%
8	katG construct2 50bp	Negative	tagaagccgctctttttcgccattggtaaaattgattgttcttatcggat	38%
9	peroxiredoxin construct1 31bp	Positive	gccgtcatttttcatagataaaacctattcc	35%
10	peroxiredoxin construct1 31bp	Negative	ggaataggttttatctatgaaaaatgacggc	35%
11	peroxiredoxin construct1 57bp	Positive	catagataaaacctattccatatagaagttttatcaattggatcgattgtgcggcgc	37%
12	peroxiredoxin construct1 57bp	Negative	gcgccgcacaatcgatccaattgataaaacttctatatggaataggttttatctatg	37%

Table 2. Oligomers constructed for EMSA study.

Electrophoretic Mobility Shift Assay

The constructed oligomers were used for binding assays to see which of the six constructed oligomers showed the best binding efficiency. Ni-NTA purified protein was diluted to known concentrations and calculated amounts were incubated with 40ng of each of the annealed oligomers. Since the protein bound DNA oligomers would run slower on a gel, the bound fraction should progress less as seen on the gels. The oligomeric construct made from KatG motif1 of length 50bp was used to standardise the concentrations for binding studies for the rest of the oligomers (**Fig 11B**). Since slightly more than 1500ng of protein was needed to bind all 40ng of protein in the first attempt, two higher and lower concentrations each of protein were incubated with the remaining 5 oligomeric constructs. All others showed lower binding efficacy than the KatG motif 1, 50 bp construct (**Figure11 A, C, D, E, F**).

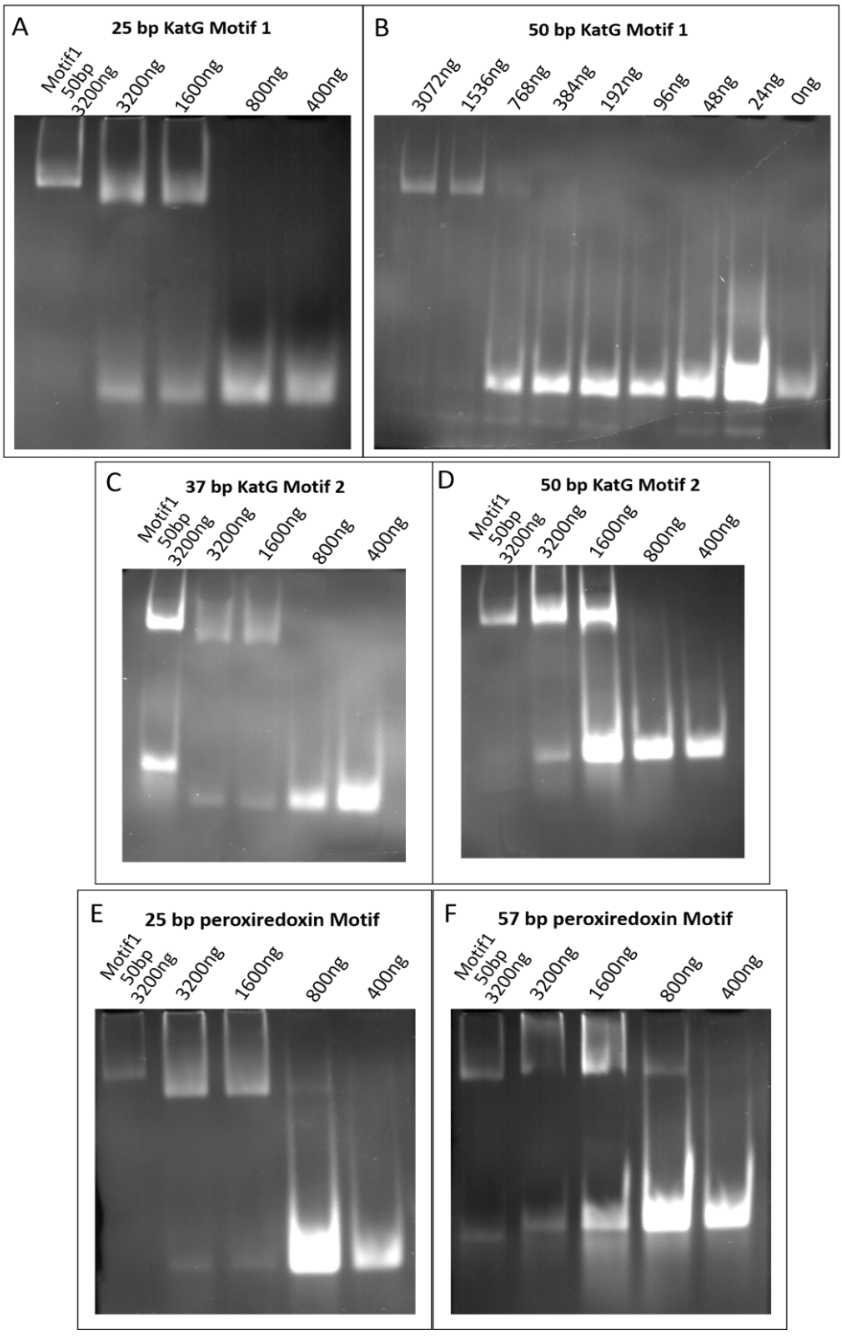

Figure 11 EMSA assays done using indicated amounts of NI-NTA purified protein incubated with 40ng of **A.** 25 bp long double stranded oligomer construct from KatG motif 1. **B.** standardisation using 50 bp long double stranded oligomer construct from KatG motif 1 **C.** 37 bp long double stranded oligomer construct from KatG motif 2 **D.** 50 bp long double stranded oligomer construct from KatG motif 1 **E.** 25 bp long double stranded oligomer construct from peroxiredoxin motif **F.** 57 bp long double stranded oligomer construct from peroxiredoxin motif

To check whether the binding efficacy of the protein product increased

after the second purification step was added, the same set of experiments were repeated with the protein purification product after FPLC (**Figure 12**). Upon using the protein concentrations standardised using NI-NTA purified product, all concentrations showed complete binding (**Fig 12A**). So, a second standardisation was done using the FPLC purification product (**Fig 12B**). The binding efficacy increased by more than two-fold, as now all the DNA was bound at 600ng of protein instead of 1500ng. The remaining 5 oligomeric constructs were tested using only the threshold concentrations of protein (**Fig 12C**) and it was found that very little binding was found in the peroxiredoxin motif constructs but none at all in the other 3 KatG motifs constructs (**Fig 12C**). The two peroxiredoxin constructs were used to do another set with increased protein concentrations (**Fig 12D**). These preliminary results may be used to conclude that the 50 bp, KatG motif 1 construct has the best binding efficacy and can potentially be used for crystallisation.

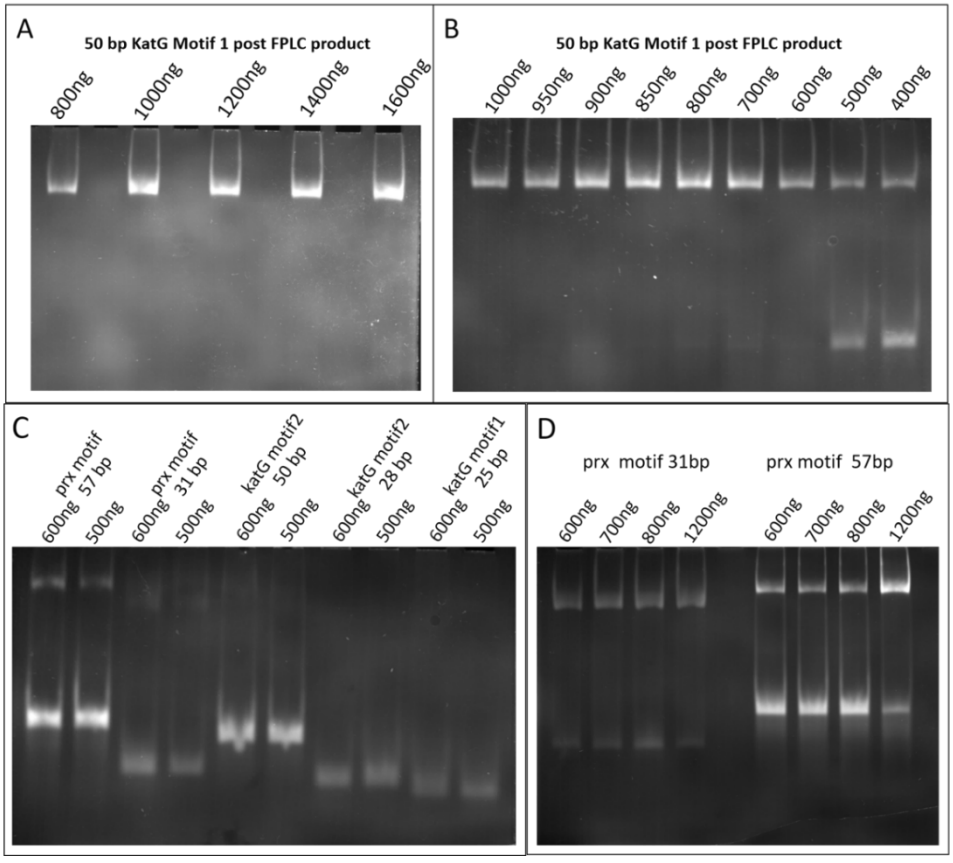

Figure 12 A. EMSA with FPLC purified protein product using previous standard of protein concentration as depicted incubated with KatG motif 1, 50 bp oligomer construct. **B.** Standardisation of protein concentration for FPLC purification product. **C.** EMSA using 40ng of remaining 5 oligomers at only the two threshold concentrations. **D.** EMSA with higher protein using only peroxiredoxin (pxr) motif constructs.

Antibiotic susceptibility assay

Since antibiotics are expected to cause sublethal doses of ROS, the cells are expected to experience oxidative stress in the presence of sublethal doses of antibiotics. To test this, six strains of *Caulobacter* were used; wild type (NA1000), two isolates of *oxyR* deletions (Δ*oxyR*1 and ΔoxyR2 and three gain-of-function mutants of *oxyR*(D4, D26, D32). The six strains were plated in five dilutions in the absence and presence of four bactericidal antibiotics (gentamicin, kanamycin, spectinomycin and ciprofloxacin). The deletion mutations showed inhibition in growth and the suppressor mutations showed better growth as compared to the wild type NA1000 (**Fig13**).

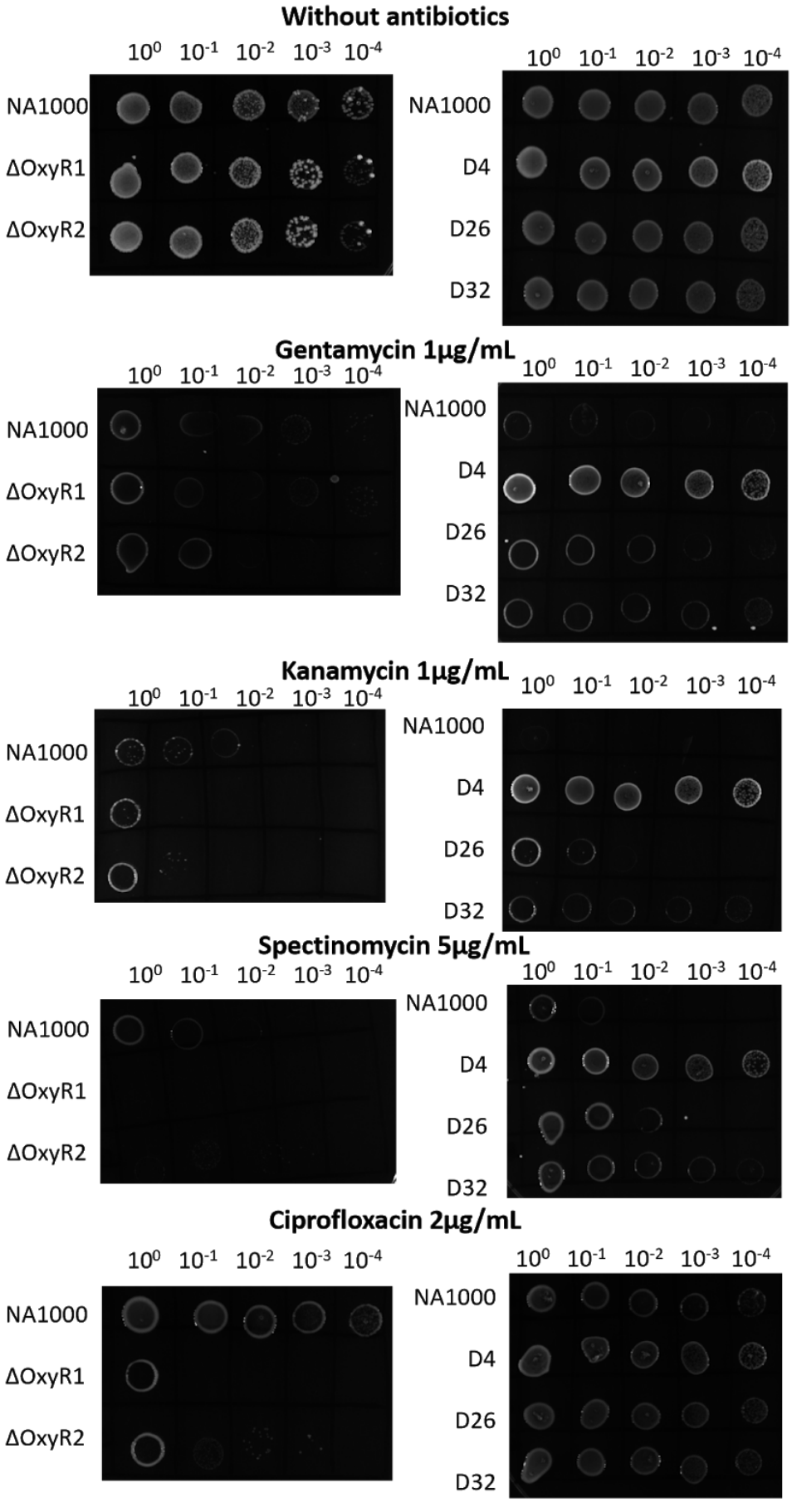

Figure 13
Plates with dilutions (as indicated) of Δ*oxyR and* gain-of-function mutatants of *oxyR* in comparison to NA1000. The antibiotic concentrations used are as represented in the figure.

Determination of Minimal Inhibitory concentration

To replicate the results found in the dilution plating experiments using growth in liquid media, first the concentration of antibiotics that showed a significant inhibition of growth in wild type was to be determined so that differential behaviour of the mutant strains could be monitored. For this, 4 or 5 concentrations of each of the antibiotics were used to grow all six strains for 24 hours. The concentrations of antibiotics at which the NA1000 showed approximately half the growth as shown in the absence of antibiotics were chosen. These concentrations were used to do the growth curves.

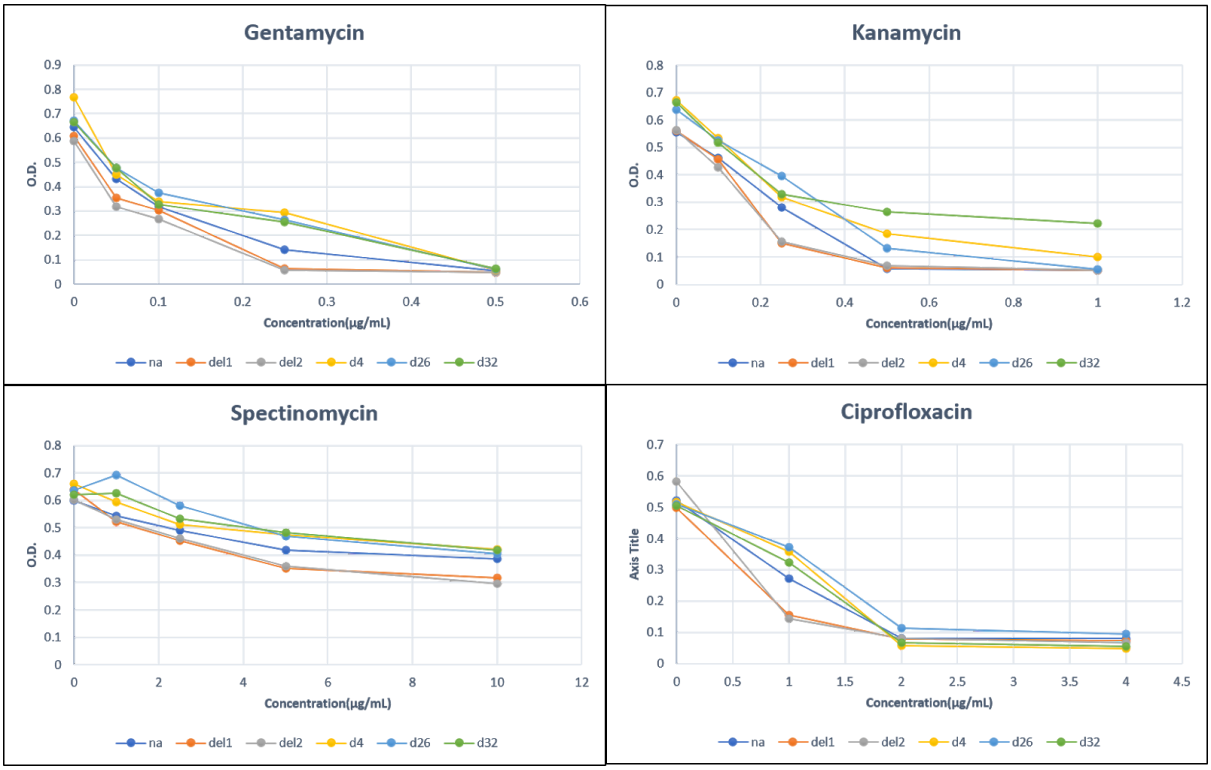

Figure14 Growth of the 6 strains NA1000(na), ΔOxyR1(del1), ΔOxyR2(del2), OxyRD4, OxyRD26, OxyRD32 across different antibiotic concentrations.

Growth in liquid media

The MIC found for the different antibiotics (1µg/mL-Gentamicin, 2.5µg/mL- Kanamycin, 10µg/mL Spectinomycin, 1µg/mL Ciprofloxacin) were used in PYE broth to monitor growth of the six strains over 12 hours. The growth seen is as shown in **Fig 15**. Even in liquid media, all six strains show similar growth in absence of antibiotics. The two deletion mutations (orange and grey) show inhibited growth as compared to the wild type NA1000 (dark blue). The three gain-of-function mutations (yellow, green and light blue) showed better growth as compared to NA1000 as predicted. However, these experiments need to be repeated to ascertain the significance of the observed results.

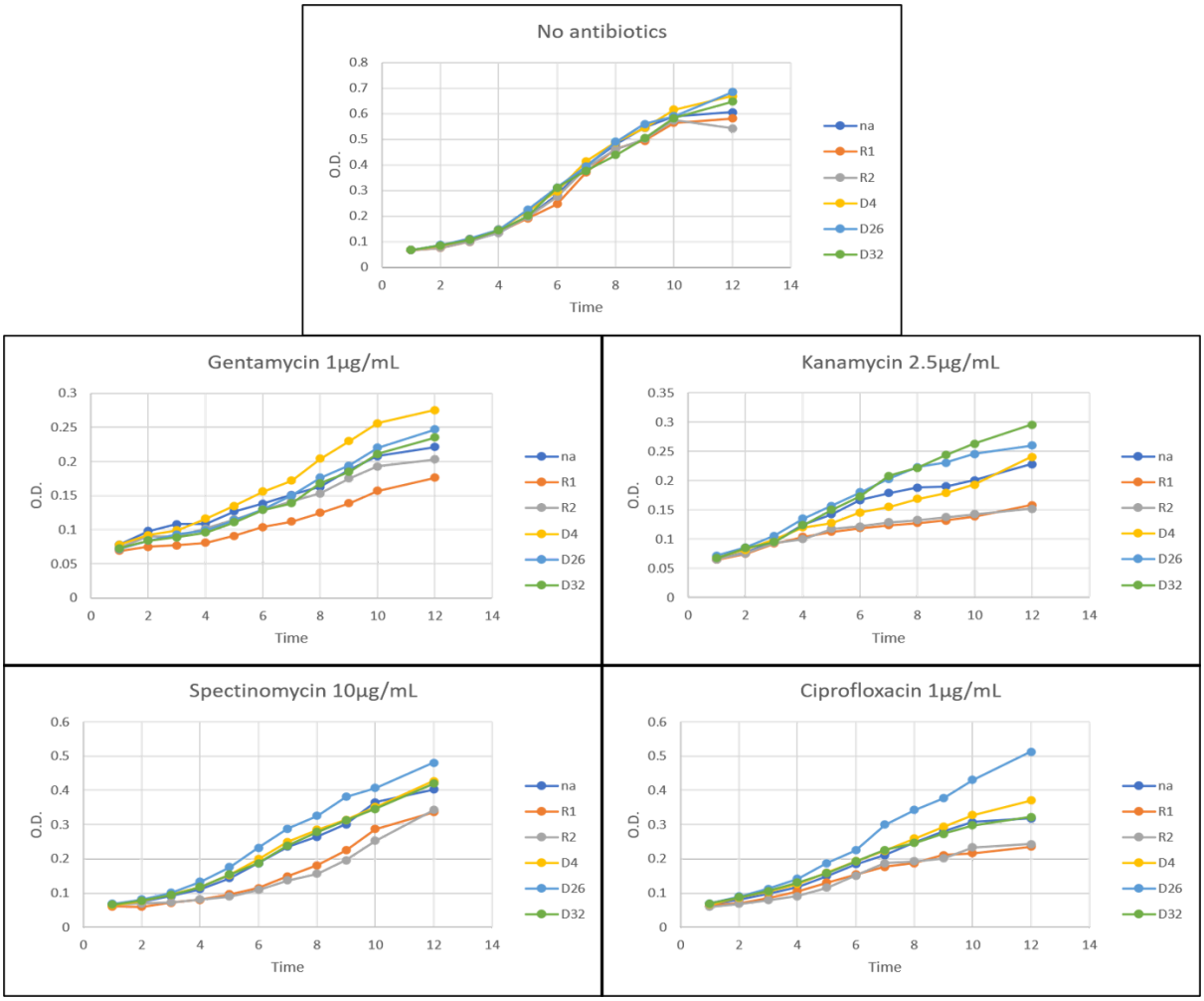

Figure 15: Growth curves of strains NA1000(na), ΔOxyR1(R1), ΔOxyR2(R2), OxyRD4, OxyRD26, OxyRD32 in the absence and presence of antibiotics

Conclusion and future direction

WT OxyR was attempted to be purified with the intention of then crystallising it in an oligomer bound state. The same was also planned to be attempted with the two suppressor mutations to see if there existed a conformational change that made the suppressor mutations constitutively activate the OxyR regulon leading to increased resistance to oxidative stress. Since the protein could not be successfully purified to the purity required for crystallisation, additional purification steps may be added to achieve purity required for crystallisation in the future. It was ascertained that the protein, even in its incompletely purified form, showed no nuclease activity and thus can potentially be crystallised in a DNA bound state.

The role of OxyR dependent oxidative stress response was studied genetically using deletions and suppressor mutations that gave results as expected from the hypothesis. The experiments can be repeated for statistical significance.

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