**Polymorphisms in efflux transporters enable rapid adaptation of cyanobacteria to antibacterial compounds**

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# Abstract

Cyanobacteria have transformed Earth's anoxic environment into an oxygen-rich atmosphere, enabling the emergence of aerobic organisms and necessitating the evolution of antioxidant defences against the reactive oxygen species (ROS) generated during oxygenic photosynthesis. Yet, the mechanisms underlying these evolutionary adaptations, especially under new anthropogenic pressures, are still unclear. Here, we applied adaptive laboratory evolution to the model cyanobacterium *Synechocystis sp.* PCC 6803, to evolve strains resistant to methyl viologen (MV), a potent ROS inducer used as herbicide in agriculture and redox mediator in electrochemistry. We successfully isolated eight strains from two phenotypically distinct parent substrains, showing up to 30-fold increased resistance compared to wild types. Genome sequencing identified several non-synonymous mutation specific to MV-resistant strains, mostly in genes encoding membrane transporters.

I would mention something here about a lack of understanding in how bacteria develop resistance to antibacterial compounds. Then you can relate this both to antibiotic production, and the adaptation of cyanobacteria to high oxygen production making it important for both medicine and ecology. I would then also mention that most model bacterial genetics uses monoploids (like E. coli) but most bacteria exhibit polyploidy and it is understudied to what extend this can influence genetic adaptation. \_\_\_This would set you up for everything else and hit all the important impactful points.

# Abbreviations

ROS: reactive oxygen species

MV: methyl viologen

BPV: biophotovoltaic

SHE: standard hydrogen electrode

**Introduction**

Life on Earth began under a reducing atmosphere (Dietrich et al., 2006) before the Archaean Eon witnessed the rise of cyanobacteria—the sole prokaryotes known to have evolved oxygenic photosynthesis (Brocks et al., 1999; Schirrmeister, Gugger & Donoghue, 2015). Such photosynthetic activity enriched the atmosphere with oxygen, necessitating that early cyanobacteria evolve antioxidant defences to mitigate the cytotoxic effects of reactive oxygen species (ROS)—a byproduct of their own metabolic innovation (Latifi, Ruiz & Zhang, 2009).

The study of oxidative stress, a state where excessive ROS production overwhelms antioxidant defences, has been advanced by using xenobiotics that simulate such conditions. Methyl viologen (MV), a bipyridinium compound known for inducing ROS, is a classic example. Initially synthesised by Weidel & Russo (1882), MV’s redox properties were recognized as biochemical tool (Michaelis & Hill, 1933) before its potent, broad-spectrum herbicidal capabilities were commercialized (Brian et al., 1958).

All of this is good for the thesis and should be saved for it, but I would not include in the paper as takes the light off the more interesting findings about adaptation and resistance. \_\_Instead here I would compare these ROS agents to antibiotics, comparing the mechanisms and discussing how their activities might be linked (e.g. Collins novobiocin paper) as well as how resistance to them is related (see other papers on how cells adapt to MV and ROS and compare with AB resistance mechansisms).

While its use as an herbicide like Paraquat has been banned due to toxicity concerns, MV persists environmentally and continues to be utilized in developing countries (Hance, Byast & Smith, 1980). In addition to agriculture, MV is commonly used as redox mediator in (bio)electrochemical systems. Thanks to its long stability and low midpoint potential of -440 mV vs SHE (Gemünde et al., 2022), MV is employed as a robust electron shuttle to reduce a wide range of inorganic and biological electron acceptors. In the presence of molecular oxygen, MV can undergo rapid reoxidation following the single-electron reduction from its divalent (MV2+) to monovalent (MV+) cationic state (Farrington et al., 1973). This results in oxygen scavenging and generation of ROS, with superoxide anions being the predominant species. In enzyme-based (*ex vivo*) bioelectrochemical devices, MV’s versatility has been demonstrated by employing it as an effective electron donor for nitrogenases, formate dehydrogenases and hydrogenases, allowing for enhanced bioelectrochemical production of ammonia, hydrogen and formate respectively (Milton et al., 2017; Jayathilake et al., 2019; Jiang et al., 2023). Among other applications, MV can be interfaced *in vivo* with non-photosynthetic bacteria for facilitating microbial reductive dichlorination of TCE (Aulenta et al., 2007), enhancing the performance of microbial fuel cells and as ROS-mediated redox inducer for electrogenetic devices (Lawrence et al., 2022).

I would link this to the paragraph above about resistance, selling cyanobacteria as a model for studying these mechanisms. Also you should stress here one advantage is that they are polyploid, just like many pathogens, and adaptation of polyploid bacteria to stress conditions is under-studied.

For these reasons, considerable research has been carried out to understand the effects of methyl viologen on photosynthetic organisms and the mechanisms underlying their inevitable evolution of resistance against it. Because of their oxygenic activity analogous to plants, their fast growth rates, genetic amenability and biotechnological potential, cyanobacteria are ideal model systems for understanding herbicide resistance (Brusslan & Haselkorn, 1988). Early experiments on the effects of paraquat on cyanobacteria indicated that the abrupt killing action of paraquat was due to it being converted into a more powerful inhibitor within the cells (Vaishampayan, 1984). Subsequent studies confirmed that MV’s cytotoxicity in cyanobacteria is mediated by its ROS-inducing activity and results in the upregulation of antioxidant pathways (Thomas et al., 1998; Campbell & Laudenbach, 1995; Li et al., 2002; Kim & Suh, 2005). Screening of mutants of the model cyanobacterium *Synechocystis* indicated that deletion of redox signalling genes such as the Serine/Threonine kinase spkB (Mata-Cabana et al., 2012) and the primary NAD kinase gene, slr1415, results in increased susceptibility to MV (Gao & Xu, 2012).

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Several studies have further identified strains resistant to MV, where altered transmembrane permeability of MV, rather than modulation of antioxidant pathways, was implicated as the mechanism of resistance (Babykin et al. 2003; Nefedova et al., 2003; Prosecka et al., 2009). However, this was not true for all of them. For example, Oh & Montgomery, (2019) found that a *Synechocystis* mutant lacking cpcG1, encoding the linker protein that connects phycocyanin to the core phycobilisome allophycocyanin, exhibited insensitivity to MV treatment. In this study, we aimed to elucidate the primary pathways cyanobacteria utilize to resist MV-induced toxicity, assess the durability of such resistance, the evolutionary processes underpinning it, and its physiological and electrochemical repercussions. By performing adaptive evolution on two substrains of the model cyanobacterium *Synechocystis sp*. PCC 6803, we isolated several resistant strains, identified the genetic mutations underlying them and characterised the trade-offs that accompany their emergence.

Mostly fine but I would reword a bit. You’re trying to understand how bacteria adapt to these stress conditions, and by now the reader should know why using MV and cyanobacteria is a good system to study this with. Explain that two sub-strains enables you to explore different evolutionary paths.

# Methods

## Strains and Growth Conditions

During this study two labtypes of the model cyanobacterium *Synechocystis* sp. PCC 6803 were used. The “Howe” labtype is a non-motile and glucose intolerant derivative of the GT-Kazusa strain that was maintained in the laboratory of Professor. Christopher J. Howe (University of Cambridge, UK). The “Nixon” type is a glucose tolerant and motile substrain that was originally a kind gift from the laboratory of Peter J. Nixon at (Imperial College London, U.K.). Cryopreserved stocks of both cultures were streaked onto BG11 Petri dishes containing 1.5% of agar. Single colonies appeared after approximately 14 days following incubation at 30C under white light illumination (100 uE). For growth curve experiments showing spontaneous evolution of MV resistance, individual colonies from the same plate were inoculated into 40 mL of BG11 medium into 50 mL Nunc flasks. Cell growth was monitored by quantifying the optical density at 750 nm using a UV-Vis Spectrophotometer (Shimadzu) using BG11 as a blank. To prevent cross-contamination, measurements were taken directly from the flask instead of a cuvette, allowing to maintain sterility throughout the whole period of the growth curve. MV stock solution were prepared by dissolving methyl viologen dichloride hydrate powder (Sigma) into autoclaved deionised water at a stock concentration of 6 mM. The solution was filter-sterilised using a 0.22 um filter and stored at -20C. For the growth curve in Figure 1 where different concentrations of MV were added into cultures, 1000x stock solution for each tested working concentrations were prepared in order to add the same volume of liquid in all conditions. The same volume of sterilised water was added for negative control conditions containing no methyl viologen. After resistant cultures appeared following MV treatment, these were spotted on BG11 plates (without MV) to isolate individual colonies, which were then inoculated in BG11 (no MV) prior to genome sequences. Parallelly, they were also inoculated on BG11 + 6 uM MV and spotted on MV containing plates to confirm that the resistance was maintained following growth in the absence of MV.

## Genome Sequencing and Analysis

For genome purification, *Synechocystis* cultures inoculated from single colonies were grown photoautotrophically (40 uE) until an OD750 of 1, and then harvested at 5000 rpm for 5 minutes. The volume of culture to pellet was calculated to obtain a total chlorophyll A mass of 40 ug, calculated according to the equation: [chlA] (mg/ml)=(OD680-OD750)x10.854 (Porra, Thompson & Kriedemann, 1989; Lea-Smith et al., 2013). The supernatant was then discarded, and the pellet was resuspended in 170 μL of freshly prepared Smoker B buffer, containing 50 mM Tris/Cl (pH 8), 50 mM EDTA, 1 % (v/v) Triton, X-100, 20 mg/mL lysozyme and 30 μL RNase ([stock]=12 mg/mL). This pre-lysis mixture was incubated at 37◦C for 1 hour. Following this, the tubes were vigorously vortexed and 20 μL of Proteinase K ([stock]= 20 mg/mL) was introduced to the solution. Another round of incubation was then carried out at 56◦C for one hour. To further homogenize the sample, glass beads were added to the solution, which was then subjected to bead beating for 5 cycles of 1 min each. In between each cycle, the tubes were cooled down on ice for 1 min. After bead beating, the mixture was centrifuged at 5000 rpm for 5 minutes. This supernatant was then carefully transferred to a NucleoSpin Tissue silica column and purification was performed according to the manufacturer instruction (Macherey-Nagel). The gDNA was then eluted by adding 50 μL of pre-equilibrated (70◦C) EDTA-free elution buffer to the column, allowing it to sit for 3 minutes, and then centrifuging at 11000 rpm for 1 minute. A subsequent addition of 50 μL of elution buffer was performed, followed by a 3-minute waiting period, and a final spin down to collect highly pure and concentrated gDNA. The concentration of purified gDNA was quantified using a nanodrop and its integrity assessed by gel electrophoresis. Library preparation and genome sequencing was performed by Novogene (UK) using Illumina PE150 technology. Results from genome extraction and QC reports are available in the supplementary information. The resulting raw reads were paired and trimmed using BBDuk and aligned to various reference genomes using bowtie2. Variant analysis was performed in Geneious and mutations were filtered and analysed using custom python scripts (scripts and all datasets available in SI).

## Oxygen Electrode and Electrochemical Characterisation

Photosynthetic oxygen evolution rates and respiration rates were determined on 2 mL cell cultures at a concentration of 4 nmol chlorophyll mL−1 harvested at an OD750 of 0.5 using a Clark-type oxygen electrode system (Oxyview, Hansatech) maintained at 30°C. Following dark adaptation (30 min), oxygen-exchange rates were recorded for 15 min at increasing light intensities (10, 25, 60, 150, 350, 900, and 2,000 µmol photons m−2 s−1) using two 9W white LED bars (RS components, 786-8979). Each light period was followed immediately by 15 min in darkness to calculate the respiration rates. The respiration rate following illumination at each light intensity was subtracted to estimate the net rate of photosynthetic oxygen evolution, accounting for oxygen uptake under illumination. To quantify the electrogenic activities of the strains, chronoamperometric measurements were undertaken at an oxidative electrode bias potential of 0.3 V vs Ag/AgCl reference electrodes (BASI) using a MultiEmStat4 potentiostat. These measurements were conducted on custom made flat photoelectrochemical cells containing indium tin oxide (ITO)-coated PET electrodes, open air platinum-coated carbon cloth cathodes, under alternating light (100 uE) and dark periods lasting two hours each. For each experiment, cultures containing a total of 250 nmoles of chlA were pelleted, washed, and loaded on the flat ITO electrodes. Cyclic voltammetries were performed using a three-electrode electrochemical cell containing a glassy carbon working electrode, platinum mesh counter and Ag/AgCl reference electrode. Scans were performed at 50 mV/s using 5 mL of supernatant obtained after centrifugation of cultures at 5000 rpm for 5 min. For anoxic scans, the solution was bubbled with nitrogen gas using a gas line tube connected to a syringe, which was bubbled for 10 min before the scan and placed in the air interface to prevent noise during the measurement.

# Results

## *Synechocystis* Evolves Spontaneous Resistance to MV During Photoautotrophic Growth

This level of detail is good for a thesis, but needs to be more succinct for a paper. I think you can cut down on a lot words in the results. E.g. for this section\_\_“In order to isolate MV-resistant mutants of *Synechocystis*, cultures were grown to mid-log phase before being exposed to between 0 and 100 uM of MV”.

To characterise the effect of MV on the growth of cyanobacteria and interrogate whether resistant strains would emerge*,* we performed a growth curve experiment starting from three biological replicates of *Synechocystis* strains divided in 4 conditions, inoculated in liquid BG11 medium, and grown under photoautotrophic conditions. When the cultures reached the mid-log phase, different concentrations of MV (0,1,10, or 100 uM) were introduced into the flasks. As anticipated, we observed growth arrest and bleached phenotypes within 24 hours of MV exposure across a range of concentrations (Fig.1A). Complete growth inhibition was observed at final concentrations as little as 1 uM, confirming the high potency of MV as an antibiotic agent. ROS quantification assays using the fluorogenic substrate DCFH-DA indicated that the wildtypes *Synechocystis* cultures treated with MV accumulated significantly higher amounts of intracellular ROS compared to non-treated cultures (Supp. Fig 1). The fold change in ROS between MV-treated and untreated cultures was significantly higher under light conditions, confirming that ROS production by MV is dependent on photosynthetic electron transport activity. However, after this expected inhibitory response, cultures treated with 1 μM and 10 μM MV, exhibited a subsequent phase of growth recovery as observed by optical density measurements and the reappearance of a bright green color within the flask. This was not observed in cultures treated with 100 μM MV. This growth resurgence following MV exposure led to further investigations to elucidate possible mechanisms for this phenomenon. To discard the possibility of the abiotic degradation of MV as a reason for the observed growth recovery, additional tests were performed. Three independent cultures originating from flasks that had shown signs of adaptation were re-diluted and exposed to a fresh stock of MV. As shown in These “adapted” cultures remained unresponsive to MV’s growth-inhibitory effects. In contrast, wild type cultures showed the expected growth inhibition effect and absence of detectable chlorophyll peaks upon re-exposure to MV. These results suggested that the observed growth recovery was not due to abiotic degradation of MV but likely indicative of a biological adaptation mechanism in *Synechocystis*, allowing it to resist MV-induced toxicity.

Figure 1) **A)** Growth curves of *Synechocystis sp.* PCC 6803 strains (“Nixon”) grown in BG11 at 30 C under constant illumination (intensity = 40 uE) in the absence (green trace) and upon addition of various concentrations of methyl viologen during exponential growth (as depicted by the legend on the top left). **B)** Schematic diagram of the experimental procedure to test the effect of selection pressure removal on the persistence of resistance. **C)** Growth curves of WT and resistant strains in the presence and absence of 6uM MV in BG11 at 30 C under constant illumination (intensity = 40 uE) **D)** Growth curves in BG11 + 6 uM MV of resistant strains that were previously (in C) cultivated without (mvR-01) and with (mvR-11) MV at 30 C under constant illumination (intensity = 40 uE). Wild-type strains were used as controls to confirm the toxicity of MV.

Again this can be one much shorter sentence, e.g.\_\_“MV-adaptation was dependent on the growth phase, with cells exposed to MV in X and Y not developing MV resistance”

Additional experiments were performed to investigate the factors contributing to the observed adaptation. A growth curve experiment in which MV was introduced at different phases of the growth (Supp Fig 7) indicated that only MV treatment during mid-log phase leads to the emergence of adapted cultures. This suggested a growth-phase-dependent mechanism of adaptation and identified the ideal conditions to evolve additional strains. Using strains of *Synechocystis* in which the prqA gene was deleted and/or later re-complemented, further experiments showed that the presence of the multidrug efflux transporter prqA (previously implicated in MV resistance) was not essential for the evolution of MV resistance, as observed by the fact that its deletion or overexpression did not affect the sensitivity to MV nor the adaptation against it (Supp.Fig.8).

Finally, we wondered whether the observed adaptability was a result of genetic changes or perhaps due to short-term adaptation mechanisms (e.g., upregulation of SOD enzymes or carotenoids passed from mother to daughter cells).

I would write this more succinctly:\_\_“We expected that such short-term adaptations would not enable cells to maintain MV-resistance after extended growth in the absence of MV (Fig. 1b). When re-exposed to MV after growth in unsupplemented media, all mutants maintained MV-resistance (Fig. 1c-d), suggesting the resistance was caused by mutations (Fig. 2).”

To test this, resistant strains were isolated into individual colonies and inoculated into liquid cultures. These clonal cultures were then divided in flasks containing no or 6 μM of methyl viologen. If the adaptation mechanisms displayed by the cultures was due to genetic mutations, we expected that removing the selection pressure of methyl viologen for some generations (growth without MV) would not affect future exposure to methyl viologen. On the other hand, if adaptation to methyl viologen was due to some generational-dependent factors, we expected that removal of selective pressure for many generations would result in cultures with loss of adaptation to methyl viologen.

As shown in Fig.1C-D, no significant difference in growth was observed adapted strains previously grown on MV (mvR-11) or in the absence of MV (mvR-01). This suggested that such adaptation mechanism persists after long-term removal of selection pressure and therefore likely due to genetic factors.

## Convergent Genetic Mutations in Methyl Viologen Resistant Strains

To understand whether genomic mutations might underlie the observed long-term adaptation of MV-resistant strains, genomic DNA from several strains that were independently adapted was purified and sequenced. When doing so, we discarded strains that lost resistance following growth in the absence of MV and selected multiple strains evolved independently during different adaptation experiments.

With the aim of maximising the genomic landscape, we adapted and sequenced *Synechocystis* strains from two different labtypes (“Howe” and “Nixon”) harbouring different genotypes and phenotypes. Alignment of the trimmed and paired reads obtained from whole genome sequencing experiments was carried out against several reference genome sequences of *Synechocystis* substrains to reduce artefacts resulting from reference genome alignments.

Make it sound like this was planned linking to the polyploidy chat in the intro. “High-coverage DNA sequencing was performed in order to identify mutations in MV-resistant strains, including any that are present as sub-populations or heterozygoyus alleles”.

For both wild-type and all resistant strains, the aligned reads covered 100% of the reference genome sequences and showed very high coverage (>300). In depth analyses of the genome sequencing results is available in the supplementary information. After having identified mutations that were also present in wild-type strains when compared to various references sequences, such “background” mutations were discarded from the variant analysis results of resistant strains. This led to the identification of resistant-specific mutations. This revealed that overall resistant strains harboured only few mutations, with most strains containing only single mutations with high variant frequencies (>75%). A shared mutational event observed in three seemingly independent MV-resistant strains ("mvR1\_Nixon", "mvR2\_Nixon", and "mvR3\_Nixon") was a leucine to proline substitution in the sll1180 gene encoding the inner permease of the S-layer type I secretion system. Interestingly, another strain (mvR6) from the same parent labtype harboured another leucine to proline substitutions in the gene sll1181, which is found in the same operon of sll1180 and encodes the translocase of the same hlyBD secretion system.

Table 1) Non-synonymous mutational events observed in MV resistant strains after removal of “background” mutations. These are the mutations found at frequencies >75% and detected irrespective of the reference genome alignment.

An additional shared mutation event was detected in the ABC transporter encoded by slr1174. In this case, 3 strains from the Howe labtype all harboured an arginine to glutamate substitution at residue 115. One strain from the Nixon labtype encoded a mutation in the same arginine residue but substituted to a histidine. This protein and the same arginine residue has previously been demonstrated to confer resistance to MV by Prosecka et al., (2009), thereby directly corroborating their findings. An additional shared phenylalanine to cysteine substitution in three strains from the Nixon labtype was observed in the gene slr1609, encoding an AMP-binding protein with predicted acyl-CoA synthetase activity. Additional unique mutations were detected in a NAD(P)H dehydrogenase subunit and an acylneuraminate cytidyltransferase as shown in Table 1. All these mutations were also confirmed by PCR amplification followed by Sanger sequencing as shown in Supp.Fig12. Interestingly, MV-resistant strains only encoded between one to a maximum of 3 (for mvR6\_Nixon) non-synonymous mutations with variant frequency greater than 75%. One outlier was mvR09\_Howe, which showed none non-synonymous mutations greater than the frequency threshold. All strains encoded additional synonymous mutations and in genomic regions outside annotated coding sequences as listed in the SI. Leveraging the high coverage obtained by our deep sequencing experiments, we calculated the frequency of each base among the reads at the locations of the mutations in the corresponding wild-type strains. As observed in Table 1, we observed a clear labtype-dependent enrichment of variants corresponding to the shared mutational events. For example, for the mutation in the sll1180 gene, we detected a polymorphic site where 10.4% of the reads from the Nixon wild type strain showed the same base that was mutated in the three Nixon MV-resistant strains. On the other hand, 100% of the reads from the Howe wild type at the same location were equal to the reference base. The same is true for the other shared mutational events in slr1174 and slr1609. This indicates that the shared mutations observed in MV-resistant strains were already present at low frequencies in the wild-type populations and became enriched following MV treatment.

## Evolution of Resistance Implies Trade-offs in Cellular Fitness

After having characterised the genotype of the isolated MV-resistant strains we performed growth curves and oxygen quantification experiments to assess the impact of the observed mutations on the physiology of the cells. As shown in Fig.3(a-c), resistant strains grew similarly to wild types in he absence of MV at low light intensities. However wild type strains grew significantly faster than resistant when the light intensity was increased to 150 µmol·s-1·m-2. As expected, in the presence of MV , wild type strains died under both illumination regimes whereas resistant strains survived the treatment and displayed slightly different growth rates between each others.

Given the central role of oxygenic photosynthesis on cyanobacterial fitness and the role of MV in oxygen scavenging, we performed oxygen electrode measurements on all sequenced wild-type and MV-resistant strains to determine the rates of oxygen evolution as a function of applied light intensity. Expectedly, as shown in Fig.3g, MV treatment resulted in significantly reduced oxygenic photosynthetic activity in wild type strains (ca. 7.6-fold and 8.8-fold reduction in “Nixon” and “Howe” strains respectively). On the contrary, unaffected rates of oxygen evolution observed in resistant strains indicated the mutants could perform oxygenic photosynthesis even in the presence of methyl viologen (Fig.3f). When resistant strains were grown in the absence of MV, they showed significantly reduced rates of oxygen evolution (Fig.3e), especially at high light intensities, similarly to the reduced growth rates displayed by resistant strains when grown at higher light intensities (Fig.3c) This indicates that evolution of resistance presents a cost in cellular fitness and explain why the conditionally beneficial mutations present at low frequency have not fixed in wild-type populations.

## Electrochemical evidence for altered MV transport in resistant strains

Genomic analyses indicated that mutant strains had mostly mutations in membrane transport components and oxygen electrode experiments demonstrated that the toxic effect of MV, which stems from its intracellular interaction with PSI, was bypassed in mutant strains. However, the findings presented so far did not explain the effects of the observed mutations and the mechanisms by which MV’s toxicity is bypassed. Leveraging MV’s membrane permeability and redox activity, we conducted a series of electrochemical experiments to characterise the electrogenic activities of both wild-type and MV-resistant strains in the presence and absence of MV. As depicted in Figure 4, when MV was absent from the electrolyte chamber (A,C), both wild-type and mutant strains produced photocurrents of similar amplitudes and profiles. This implies that the emergence of methyl viologen resistance does not influence the cell’s electrogenic activity in the absence of MV. However, upon addition of 6 μM MV into the anodic chamber, the electrogenic profiles from the wild-type (B) and MV-adapted strains (D) displayed significant differences. During the initial three light cycles, wild-type strains generated large photocurrents, reaching amplitudes nearly three times greater than those observed without the presence of MV. Subsequently, the electrogenic activity decreased, and the photocurrent profiles were no longer detectable. By the experiment’s end, the formerly green biofilm of cells adhered to the electrode turned white, suggesting cell toxicity. Conversely, the MV-adapted strains, when subjected to MV, produced photocurrents similar to those recorded in the absence of MV. Unlike the wild-type cells exposed to MV, the biofilms derived from the MV-adapted strains remained green and viable until the conclusion of the experiment. culture’s supernatant. However, given the low concentrations of MV used to treat cultures, we were not able to confidently estimate MV’s concentration spectroscopically due to the low signal-to-noise ratio. For this reason, we employed cyclic voltammetry (CV), which is a much more sensitive technique, to quantify the redox profiles in the cultures following MV treatment.

Cultures of wild types and resistant strains were treated with MV and grown photoautotrophically. After 4 and 15 hours post treatment, the cells were centrifuged and the concentration of extracellular MV was quantified by performing CVs on the supernatant. At a potential of ca. -0.5 V vs Ag/AgCl, MV reduces molecular oxygen, so we performed CVs with and without nitrogen purging to use the oxygen reduction peak as an estimate of MV2+ concentration. As shown in Fig.4(e-n), the CVs showed that indeed the supernatant from all cultures showed characteristic MV redox peaks, indicating that MV has not been degraded. As observed by the reduction in the peaks in the presence of oxygen at 4 and 15 hours, the concentration of MV2+ in the supernatant from wild-type cultures decreased over time. This indicates that in these cultures MV2+ is reduced into its membrane permeable form, goes intracellularly and thus MV2+ in the supernatant disappears. On the contrary, the oxygen reduction peaks catalysed by MV2+ in the supernatant from all resistant cultures remained almost unchanged. This confirms that resistant strains display a reduced intracellular accumulation of MV.

**Discussion**

In this study, we demonstrated that *Synechocystis* cells can spontaneously evolve resistance to the bactericidal effects of methyl viologen. Despite wild type cultures bleached after treatment with MV at concentrations as low as 1 μM, emergence of green and viable cultures was observed during liquid cultivation after a period of apparent growth inhibition. These adapted cultures were insensitive to addition of freshly prepared MV, indicating that such growth resurgence was a result of biological evolution rather than abiotic degradation of the chemical, as often observed in the case of β-lactam antibiotics (Brouwers et al., 2020). Emergence of spontaneous mutants was observed for both “Howe” and “Nixon” *Synechocystis* substrains, but only occurred when MV was added during mid-log growth and at concentrations lower than 100 μM. Given the detection of MV-resistance alleles occurring at low frequencies even within unadapted cultures, the resurgence of proliferating cultures following the phase of apparent growth inhibition can be explained as the period during which the majority of cells died, while a few carrying the beneficial polymorphisms resisted and resumed their growth, eventually reaching a state where the cultures regained visible greenness. Babykin et al., (2003) and Prosecka et al., (2009) previously reported spontaneous MV-resistant mutants of *Synechocystis*, which were obtained by successive rounds of re-streaking on solid plates. Not only we recapitulated the same mutations observed by Prosecka et al., (2009), but we expand on their knowledge by identifying the conditions in which such adaptive evolution can occur in liquid cultures during photoautotrophic cultivation. This can be advantageous for screening cyanobacterial strains robust to oxidative stress in Adaptive Laboratory Evolution (ALE) experiments using liquid-based continuous cultivation systems and industrial photobioreactors, as demonstrated by similar efforts aimed at enhancing photosynthesis (Dann et al., 2021; Sun et al., 2023) .

Given that MV treatment can significantly alter gene expression profiles, antioxidant responses (Li et al., 2002; Kim & Suh, 2005) and the secretome (Ongley, Pengelly & Neilan, 2016; Hu et al., 2022) of cyanobacteria, we wondered whether the observed adaptation to MV was consistent with a genetic or an epigenetic inheritance mode of resistance (Adam et al., 2008). As revealed by the persistence of resistance following long-term cultivation in the absence of selective pressure, our results suggested that resistance is accompanied by changes in the DNA sequence of cyanobacterial genomes. Genomic sequencing experiments and analyses further corroborated this hypothesis by identifying a few mutations specific to MV-adapted strains. Although there is no single mechanism to obtain antimicrobial resistance, generally this is achieved via reduction in intracellular drug accumulation (reduced permeability and antibiotic efflux), modification or alteration of the bacterial antibiotic target, modification or destruction of the drug itself, and bypass of whole metabolic pathways (Darby et al., 2022). Our sequencing results indicated that all resistant strains, with the exception of one (mvR\_09) encoded non-synonymous mutations in membrane transporter components. Corroborating previous studies (Nefedova et al., 2003; Hu et al., 2022; Ongley, Pengelly & Neilan, 2016; Prosecka et al., 2009), our findings suggest that the mode of MV resistance in *Synechocystis* is via reduction in intracellular drug accumulation. For the first time, we sequenced whole genomes and identify variants in multiple independently adapted strains from two genotypically and phenotypically distinct parent substrains. All mutants with the exception of mvR\_09 encoded mutations in either the type I S-layer secretion system or the ABC transporter slr1174. Interestingly, Prosecka et al., 2009 isolated MV-resistant strains with mutations in the same conserved R115 residue of slr1174 as we observed for strains mvR6,10,11,12, thereby directly hinting at slr1174 role in MV resistance. By performing site-directed mutagenesis they confirmed the importance of this residue in conferring resistance to MV and suggested that changes in this residue determine the substrate specificity of the transporter. Four other mutants encoded single nucleotide polymorphisms in the sll1180-sll1181 operon, which encodes the type I system that secretes the S-layer protein (Agarwal et al., 2018) and has been implicated in antibiotic and acid stress tolerance (Uchiyama et al., 2019). Additionally, three strains from the motile and glucose-tolerant “Nixon” labtype encoded a mutation in the slr1609 gene, encoding a long-chain acyl-CoA synthetase (von Berlepsch et al., 2012). Given that ROS can cause damage to lipids via peroxidation reactions (Bus, Aust & Gibson, 1974), such mutation might further help the cells to cope with MV-induced toxicity. Unlike Nefedova et al., (2003) we did not find mutations in the MATE-type efflux transporters PrqA, which was previously implicated in controlling MV resistance in *Synechocystis*. In addition, adaptive evolution experiments using *Synechocystis* strains that lacked or overexpressed the prqA gene (Supp.Fig.8), indicated that this gene, although might be important for susceptibility to sublethal MV concentrations, is not essential for the long-term evolution of MV resistance.

At first, we were surprised to observe such a high frequency of shared mutational event in seemingly independently evolved strains. Given that adaptive evolution was performed in different flasks (which remained always sealed post MV treatment) inoculated from different colonies and subjected to independent MV treatments, it is unlikely that such shared mutational events were a result of cross-contamination. A principal component analysis (Supp.Fig.12) which considered all mutations (also in non-coding regions and non-synonymous) indeed discarded this possibility. In fact, strains that harboured the shared mutational events differed significantly between each other when all mutations were considered and therefore are likely to be different substrains that obtained the same mutations by convergent evolution. The alternative explanation is that those mutations that converged in some substrains and not others were already present at low frequency in wild type strains and were enriched in MV-resistant strains by natural selection following MV treatment. This argument is favoured also by the fact that we detected the same mutations as those observed following ALE to enhance acid stress (Uchiyama et al., 2015) and similar spontaneous MV-resistant mutants by (Prosecka et al., 2009). The variant frequency analysis performed in wild type strains (Table1) and Sanger sequencing chromatograms (add fig X) indeed confirmed that the beneficial mutations observed in MV resistant mutants were also present at low frequencies in wild type, unadapted cultures. Given that genome sequencing and colony PCRs were performed on isolated, clonal colonies, this suggests that there are polymorphic sites in the genome of individual cells. Indeed, single cyanobacterial cyanobacterial cells have been shown to possess non-equivalent genomes, which was true not only for the oligoploid *Synechococcus elongatus* PCC 7942 but also for the polyploid *Synechocystis*, which contains 10–20 genome copies per cell (Soppa, 2021). Genome polyploidy could allow for a larger genetic "playground," wherein certain gene copies can undergo mutations without drastically compromising overall cellular function. This redundancy may have been evolutionarily favoured, especially in changing environments where adaptability is crucial. Here for the first time we present evidence that such genomic polymorphism in clonal populations of cyanobacteria might have a biological function in enabling balancing selection mechanisms to adapt to changing environments. Further experiments using single cell genome sequencing and RNA-seq will be useful to further corroborate this mechanism.

After having identified that genetic mutations underlying MV resistant strains, characterisation experiments were performed to assess the trade-offs which often accompany the evolution of antibiotic resistance (Phan & Ferenci, 2017). All isolated MV-resistant strains showed reduced rates of oxygen evolution compared to wild types in the absence of MV. This suggests a negative impact of MV resistance on the fitness of cyanobacterial cells and explains why the observed, conditionally beneficial mutations have not fixed in wild type populations. We further employed electrochemical techniques to probe the effect of MV evolution on the extracellular electron transfer of cyanobacteria and to quantify MV’s transport across the cell.

As shown by chronoamperometries in the presence of MV in BPV devices containing wild type strains, MV dramatically increased the photocurrent, confirming its role in accepting intracellular photosynthetic electrons to reduce extracellular electrodes. However such large photocurrent were short lived and the biofilms adhered to the electrodes were bleached, suggesting that extracting electrons from PSI with MV inevitably leads to ROS production and cell death. On the contrary with the exception of strains mvR1-2-3 (see Supp.Fig.5), MV-resistant strains in the presence of MV displayed similar photocurrents than those observed in its absence and remained viable at the end of the experiment. This suggests that MV-resistant strains of *Synechocystis* achieve resistance primarily by abolishing intracellular accumulation of MV or by inhibiting its redox activity (e.g. by chemical degradation). Cyclic voltammetries performed on the supernatant of cultures after 4 or 15 hours post MV treatment indicated that the extracellular concentration of MV in MV-resistant cultures remains unchanged over time, whereas it declines (as MV penetrates intracellularly) in wild types. This finding provides direct evidence that is the reduction of intracellular MV accumulation the mode of resistance, corroborating the genomic observation, This is a double-edged sword. On one hand, these strains can survive in MV-rich environments, which is advantageous for their ecological competitiveness and potential biotechnological applications. On the other hand, the very mechanism that confers resistance— the prevention of intracellular MV accumulation—negates the utility of MV as a redox mediator for these strains. In essence, if MV cannot accumulate intracellularly, it cannot serve its role in shuttling electrons from intracellular metabolic pathways to the electrode. This means that while MV-resistant strains can survive in environments where MV is present, they cannot benefit from the enhancement of photocurrents that MV typically offers.

Despite this, the MV-resistant strains isolated in this study might be useful for future biotechnological applications. For example, MV-resistant strains could be used in co-culture based bioelectrochemical devices in which cyanobacteria provide sugars to electroactive heterotrophic bacteria in the same chamber (Zhu et al., 2022), where MV could be added as a redox mediator to enhance the performance without compromising the photosynthetic activity of the primary producers. In addition, MV can be employed as an electron mediator to supply external electrons for the turnover of intracellular nitrogenase in bioelectrochemical devices containing *Synechococcus elongatus* PCC 7942 for bioelectrochemical nitrogen fixation (Dong et al., 2021). However, MV toxicity was reported as a limiting factor and, based on these findings exposure to MV will inevitably lead to reduction of intracellular MV and thus MV-mediated nitrogenase turnover after long-term operation of such devices. Expression of MV-resistant genes like the mutated slr1174 protein driven under a promoter specific to photosynthetically active cells and not within heterocysts could allow to introduce MV in cultures of filamentous diazotrophic cyanobacteria. The cells could still perform photosynthesis being resistant to MV, but MV could shuttle into heterocysts cells, which are anaerobic, to significantly enhance biological nitrogen

Future work should also determine whether enhanced efflux of methyl viologen as displayed by the strains isolated here, also leads to enhanced efflux of additional compounds in a generalised way. If so, this approach would be beneficial to enhance the secretion of target compounds for photobioproduction.

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