**Polyploid cyanobacterial genomes provide a reservoir of mutations allowing rapid evolution of herbicide resistance**   
  
Alberto Scarampi, Joshua M. Lawrence, Paolo Bombelli, Darius Kosmützky, Jenny Z. Zhang, Christopher J. Howe  
  
 Department of Biochemistry, University of Cambridge, UK  
 Yusuf Hamied Department of Chemistry, University of Cambridge, UK  
 Corresponding author(s) Lead contact: ch26@cam.ac.uk

**Abstract**

Adaptive mechanisms in bacteria, which are widely assumed to be haploid or partially diploid, are thought to rely on the emergence of spontaneous mutations or lateral gene transfer from a reservoir of pre-existing variants within the surrounding environment. These variants then become fixed in the population upon exposure to selective pressures. Here, we show that multiple distinct wild type substrains of the highly polyploid cyanobacterium *Synechocystis sp.* PCC 6803 can adapt rapidly to the potent herbicide methyl viologen. Genome sequencing revealed that the mutations responsible for methyl viologen (MV) adaptation were already present prior to selection in the genomes of the unadapted parental strains at low allelic frequencies. This indicates that chromosomal polyploidy in bacteria can provide cells with a reservoir of conditionally-beneficial mutations that can become rapidly enriched and fixed upon selection. MV-resistant strains performed oxygenic photosynthesis less efficiently than wild-types when MV was absent, suggesting trade-offs in cellular fitness associated with the evolution of MV resistance, and a possible role for balancing selection in the maintenance of these alleles under ecologically-relevant growth conditions. The mode of resistance was confirmed to be reduced intracellular accumulation of MV. Our results indicate that genome polyploidy plays a role in the rapid adaptation of some bacteria to stressful conditions which may include xenobiotics, nutrient limitation, environmental stresses and seasonal changes.

# Introduction

Bacteria constantly adjust their physiological state to survive in changing environmental conditions. Alterations in cellular activity can be temporary, mediated by differential genetic and metabolic regulation, or more permanent, resulting from the establishment of genetic mutations within bacterial genomes. The latter process, known as adaptative evolution, depends on the availability of beneficial variants and their fixation within the population1. As currently understood, there are multiple ways of obtaining beneficial variants, primarily through spontaneous mutation, or lateral gene transfer between different organisms2. In general, adaptation to new environments occurs in two distinct ways: selection on pre-existing genetic variation or selection on new mutations (i.e. spontaneous mutagenesis). The former leads to faster evolution, and may result in the fixation of recessive alleles, or multiple alleles with individually small effects3.

Much of the attention on understanding the mechanisms of microbial adaptation has concentrated on the effect of horizontal gene transfer4 and the use of model haploid/diploid strains such as *Escherichia coli*5. However, axenic cultures of isogenic bacterial populations with no access to environmental reservoirs of beneficial alleles can rapidly evolve adaptive phenotypes6, and many phylogenetically diverse prokaryotic strains exhibit polyploidy7,8, which can result in heterogeneity among copies of their chromosomes7,9. However, the physiological role and evolutionary consequences of genome ploidy in bacteria remain largely unknown.

Cyanobacteria exhibit a high degree of genome polyploidy, with the model freshwater strain *Synechocystis sp*. PCC 6803 containing more than 100 genome copies during exponential phase10. The ecological success of cyanobacteria is demonstrated by their diversity and ubiquity, being found in most environments on earth11–13. This has necessitated their adaptation to various environmental conditions, especially those which influence the formation of harmful reactive oxygen species (ROS). Cyanobacteria generate oxygen via photosynthetic water oxidation, which increases their exposure to ROS. If left unchecked ROS can lead to oxidative stress: a physiological state characterised by cellular toxicity14. For these reasons, cyanobacteria are ideal model organisms for studying genome polyploidy as it relates to microbial adaptation.

Methyl viologen (MV), a bipyridinium compound known for inducing ROS, is a xenobiotic compound widely employed to study oxidative stress responses. Initially synthesized in 188215, MV’s redox properties were recognized as a biochemical tool16 before its potent, broad-spectrum herbicidal capabilities were commercialised17. More recently, MV has been employed in (bio)electrochemical systems as a robust extracellular electron shuttle to reduce a wide range of inorganic and biological electron acceptors18–23. Whilst toxicity concerns have led to MV being banned in some countries, it persists environmentally and continues to be utilised in many countries24.

MV exists in the divalent MV2+ state under ambient conditions but can be readily reduced by the photosynthetic electron transport chain to the monovalent (MV•+) state. In the presence of molecular oxygen, MV•+ undergo rapid reoxidation to MV2+ 25, generating (predominantly) superoxide in the process26. This redox-cycling ROS generation results in cytotoxicity and the upregulation of antioxidant pathways27–30. Screening of mutants of the model cyanobacterium *Synechocystis* indicated that deletion of redox signalling genes such as *spkB*, encoding a serine/threonine kinase, and *slr1415*, encoding the primary NAD kinase, results in increased susceptibility to MV31,32. Several studies have further identified strains resistant to MV33–36, with most of these studies implicating altered transmembrane permeability of MV as the mechanism of resistance33–35.

In this study, we aimed to understand the mechanisms allowing cyanobacteria to adapt rapidly to MV-induced oxidative stress. We performed adaptive evolution on two wild type substrains of the model cyanobacterium *Synechocystis sp*. PCC 6803, enabling us to explore multiple evolutionary paths to resistance. We isolated several resistant strains, identified the genetic mutations underlying them and observed that those likely to be responsible for MV resistance were already present, at low frequencies, in wild-type parent strains. Our results indicate that highly polyploid bacteria may sustain a pool of mutant alleles enabling rapid response to environmental stresses.

# Results

## *Synechocystis* adapts rapidly to MV during photoautotrophic growth

In order to adapt *Synechocystis* toMV, cultures were grown in the standard photoautotrophic medium BG11 until mid-log phase before being exposed to between 0 and 100 μM MV (Figure 1A). As anticipated, we observed growth arrest and bleached phenotypes within 24 hours of MV exposure across a range of concentrations (Figure 1B). Complete growth inhibition was observed at MV concentrations as low as 1 μM, confirming its high potency. ROS quantification assays using the fluorogenic substrate DCFH-DA indicated that the wild-type *Synechocystis* cultures treated with MV accumulated significantly higher amounts of intracellular ROS than non-treated cultures (Figure S1). The fold-change in ROS between MV-treated and untreated cultures was significantly higher under illumination, confirming that ROS production by MV is dependent on photosynthetic electron transport activity.

After this expected growth inhibition, 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 colour within the flask. This was not observed in cultures treated with 100 μM MV. To test if growth recovery was due to degradation of MV, three independent cultures originating from flasks that had shown subsequent growth in the presence of MV were subsequently re-diluted and exposed to 6 μM MV from a freshly prepared stock. These “adapted” cultures remained unresponsive to MV’s growth-inhibitory effects. In contrast, wild-type cultures showed the expected growth inhibition (Figure S2) and bleaching (Figure S3) effect. These results demonstrate that the observed growth recovery was not due to degradation of MV and indicate a biological adaptation mechanism in *Synechocystis*, allowing it to resist MV-induced toxicity.

Additional experiments suggested that MV-adaptation was dependent on the growth phase, with cells exposed to MV in lag and stationary phases not developing MV resistance (Figure S4).

In order to confirm if the adaptability of *Synechocystis* to MV was a result of genetic changes rather than short-term adaptive mechanisms facilitated by phenotypic plasticity (e.g. upregulation of superoxide dismutase enzymes or antioxidant pigments passed from mother to daughter cells), we re-exposed adapted cultures to MV after a week of growth in the absence of MV (Figure 1C). As shown in Figure 1D-E, when re-exposed to MV after growth in unsupplemented medium, all mutants maintained MV-resistance, suggesting the resistance was caused by genetic mutation.

A diagram of a cell cycle

Description automatically generated with medium confidence

Figure 1. A) Schematic diagram for the adaptive laboratory evolution of MV-resistant strains. B) Growth curves of *Synechocystis sp.* PCC 6803 wild type (“Nixon”) strain grown in BG11 at 30 ℃ under constant illumination (40 μmol⋅s-1⋅m-2) in the absence or presence of various concentrations of methyl viologen added during exponential growth. C) Schematic diagram of the experimental procedure to test the effect of selection pressure removal on the persistence of resistance. D) Growth curves of WT and resistant strains in the presence or absence of 6 μM MV in BG11 at 30℃ under constant illumination (40 μmol⋅s-1⋅m-2) E) Growth curves in BG11 + 6 μM MV of resistant strains that were previously (in C) cultivated without (mvR-+) and with (mvR++). MV at 30℃ under constant illumination (intensity = 40 μmol⋅s-1⋅m-2). Wild-type strains were used as controls to confirm the toxicity of MV.

## Methyl viologen resistant strains show convergent mutations

The previous experiments indicated that the observed long-term adaptation of MV-resistant strains was due to mutations. To identify mutations that may be responsible, genomic DNA from several independently-adapted strains was purified and sequenced. These included strains originating from wild type parental ones from two different laboratories (referred to as “Howe” and “Nixon” wild types) which had ultimately been derived from the same environmental isolate of *Synechocystis* sp. PCC 6803 (Figure 2A, Figure S5). DNA from the parental strains (wt) was also sequenced, as well as DNA from wild-type strains subjected to the same long-term ALE conditions but in the absence of MV (wt-MV). High-coverage DNA sequencing was used, to allow identification of mutations at low frequencies in the DNA from individual colonies. For both wild-type and all resistant strains, the aligned reads covered 100% of the reference genome sequences and showed very high coverage (>300) (Table S1). In-depth analyses of the genome sequencing results are available in the supplementary information (Table S1-3, Figure S6-9). “Background” mutations that were present with variant frequencies >0.75 in wild-type strains in comparison to various reference sequences as well as in resistant strains, were discarded from the variant analysis performed on the results of resistant strains. Variant analysis revealed only a few segregated (i.e. with variant frequencies >0.75) non-synonymous mutations in resistant strains. A number of these mutations were observed independently in different resistant strains. As shown in Figure 2B and Table 1, three independently adapted MV-resistant strains (“mvR01\_Nixon”, “mvR02\_Nixon”, and “mvR03\_Nixon”) shared a mutation in the *hlyB* gene (*sll1180*) resulting in a L139P substitution in the inner permease of the S-layer type I secretion system. Interestingly, another strain (mvR06) derived from the same parental strain displayed another L115P substitution in HlyD, an ABC translocase encoded by *sll1181* in the same *hly* operon, which also forms part of the HlyBD secretion system. An additional shared mutation event was detected in *slr1174*, encodingan ABC transporter. In this case, 3 strains from the Howe background all harboured the same mutation, resulting in an R115G substitution. One strain from the Nixon background had a mutation in the same codon, instead leading to a R115H substitution. A mutation in this arginine residue has previously been demonstrated to confer resistance to MV33. An additional shared mutation in three strains from the Nixon background was observed in the *aas* gene (*slr1609*) resulting in an F255C substitution in the acyl-acyl carrier protein synthetase involved in membrane lipid translocation37. However, the same mutation was also observed (Table S4) in the corresponding wild-type strain subjected to ALE in the absence of MV (wt-MV\_Nixon), indicating that the establishment of such variant was not specific to MV treatment but rather to long-term laboratory cultivation. An additional unique mutation was detected in locus *slr0262*, which codes for a hypothetical protein whose expression was found to be downregulated in mutants lacking a specific ferredoxin gene under oxidative stress conditions38. Individual MV-resistant strains exhibited only 1-3 non-synonymous chromosomal mutations with variant frequency greater than 0.75. Interestingly, mvR09\_Howe exhibited no chromosomal non-synonymous mutations above the frequency threshold. A similar analysis of the large plasmids pSYSM, pSYSA, pSYSX, and pSYSG was performed, with non-synonymous mutations being identified only in pSYSM for the strains mvR10-12 (Figure S10). However, these must be regarded as putative mutations because the sequencing error rate of this particular plasmid for the parental “Howe” wild-type strain was above the threshold required for confident variant A diagram of a circle with different colored circles

Description automatically generated with medium confidenceanalysis.

**Figure 2.** A) Diagram showing the history of adaptive laboratory evolution of the *Synechocystis* strains presented in this study B) Comparative genomic analysis of multiple MV-R strains. Circular representation of genomes with concentric circles corresponding to different strains. White ticks denote single nucleotide polymorphisms (SNPs) across both coding and non-coding sequences, while black lines indicate non-synonymous mutations within coding sequences (CDSs). All depicted mutations have been filtered to exclude those present in respective background strains and those with variant frequencies <0.75. C) Percentage of low frequency (<0.25) variants (i.e. polymorphisms) compared to all detected variants across all sequenced strains. All mutation types were considered, including synonymous and non-synonymous mutations in protein coding sequences, as well as mutations in non-protein coding sequences. D) Percentage of high frequency (>0.75) variants (i.e. segregated mutations) compared to all detected variants across all sequenced strains considering both coding and non-coding sequences and all mutation types (even those with no effects on protein sequences). Histograms displaying the distribution of number of variants as a function of variant frequencies at multiple bin sizes are shown in Figure S11-13.

**Table 1.** Table of segregated (variant frequency >0.75) non-synonymous mutations resulting from comparative genomic variant analysis of MV resistant strains.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Gene** | **Locus** | **Strains** | **Description** | **Variant frequency** | **Variant frequency (in wild type)** | **Mutation** |
| *hlyB* | sll1180 | mvR01\_Nixon, mvR02\_Nixon, mvR03\_Nixon | peptidase domain-containing ABC transporter (HlyB) | 1, 0.99, 1 | Howe: 0 Nixon:0.10 | L139P |
| *hlyD* | sll1181 | mvR06\_Nixon | ABC family efflux transporter periplasmic adaptor subunit (HlyD) | 0.83 | Howe: 0 Nixon:0 | L115P |
| *evrB* | slr1174 | mvR10\_Howe, mvR11\_Howe, mvR12\_Howe | ABC-2 family transporter protein (EvrB) | 1, 1, 1 | Howe: 0.067 Nixon:0 | R115G |
| mvR06\_Nixon | 0.81 | Howe: 0 Nixon:0 | R115H |
| n.a. | slr0262 | mvR10\_Howe | hypothetical protein | 0.84 | Howe: 0 Nixon:0 | A99P |

All MV-adapted strains showed additional synonymous mutations alongside mutations in genomic regions outside of annotated coding sequences. 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. Strikingly, we observed that in all cases where a given mutation was found in more than one independently-adapted strain, the parent strain harboured the same allele at a lower frequency (Table 1). For example, for the mutation found in the *hlyB* gene (*sll1180*) in ~100% of reads from three different MV-resistant strains derived from the Nixon wild-type (mvR01, mvR02, and mvR03), 10.4% of the reads from the Nixon wild-type strain showed the same mutation. On the other hand, no reads from the Howe wild-type strain showed the presence of this allele, and none of the adapted strains derived from it showed mutations in *hlyB* (*sll1180)*.The same was true for the other shared mutational events in *evrB* (*slr1174*). This indicates that the shared mutations observed in MV-resistant strains were already present at low frequencies in the respective wild-type strain and became enriched following MV treatment.

Further analysis was performed in order to determine if MV-adaptation was characterised by a global change in nucleotide variant frequencies, including in non-synonymous mutations, mutations outside of protein-coding regions, and mutations with variant frequencies <0.75. To do this, all variants with frequencies above 0.01 (including those found in wild-type sequences) were considered. The variant frequency distributions of all strains were then compared at various binning parameters (Figure S11-13), which revealed an inverse relationship between number of variants and variant frequencies similarly across all strains. Strains harboured between 111 and 218 total mutations, of which on average (across all strains) 40% of those maintained as low frequency polymorphisms (frequency < 25%) and 10% as segregated variants (frequency >75%). Interestingly, low-frequency variants were diminished whereas high-frequency variants were enriched in all MV-resistant mutants relative to their wild-type parent strains (Figure 2C-D). This suggests MV adaptation is characterised by the fixation of polymorphisms into fully segregated mutations, corroborating the observation that wild type strains maintain a pool of low-frequency variants to enable rapid adaptation to selective pressures.

## Methyl viologen resistance is associated with trade-offs in cellular fitness

Having characterised the genotype of the isolated MV-resistant strains, we performed growth curves to assess the impact of the observed mutations on the fitness of the cyanobacteria. As shown in Figure 3A, resistant strains grew similarly to wild-types in the absence of MV at low light intensities. However, wild-type strains grew faster than resistant ones when the light intensity was increased to 150 μmol⋅s-1⋅m-2 (Figure 3B). 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 other (Figure 3C-D).

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 (Figure 3E-F). Expectedly, 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). By contrast, rates of oxygen evolution were unaffected by MV in the resistant strains, indicating they could perform oxygenic photosynthesis even in the presence of methyl viologen (Figure 3F). However, when resistant strains were grown in the absence of MV, they showed significantly reduced rates of oxygen evolution compared to wild-type (Figure 3E), especially at high light intensities, similarly to the reduced growth rates displayed by resistant strains when grown at higher light intensities (Figure 3C). This indicates that MV A collage of graphs

Description automatically generatedresistance presents a cost in cellular fitness in the absence of MV.

Figure 3. **A)** Growth measurements of WT and mvR *Synechocystis* strains in BG11 at 30 °C in continuous low light (40 μmol⋅s-1⋅m-2) in the absence of MV. **B)** Growth measurements of WT and mvR *Synechocystis* strains in BG11 at 30°C in continuous high light (150 μmol⋅s-1⋅m-2) in the absence of MV. **C)** Growth measurements of WT and mvR strains in BG11+ 6 μM at 30°C in continuous low light (40 μmol⋅s-1⋅m-2). **D)** Growth measurements of WT and mvR strains in BG11+ 6 μM at 30 °C in continuous high light (150 μmol⋅s-1⋅m-2). **E)** Net oxygen evolution rate (dark-adjusted) as a function of light irradiance for wt and mvR strains in BG11. **F)** Net oxygen evolution rate (dark-adjusted) as a function of light irradiance for wt and mvR strains in BG11+6 μM. **G**) Intracellular ROS content across all *Synechocystis* strains as quantified by the ROS-sensitive fluorogenic substrate DCFH-DA. Prior to incubation with DCFH-DA, *Synechocystis* strains growing at an OD750 = 0.5 were treated with 6 μM methyl viologen for 15 hours in the light (intensity=40 μmol⋅s-1⋅m-2). The bulk fluorescence intensity values were normalised to the OD750 and error bars represent standard deviations from the mean for three biological replicates. H) Concentration of methyl viologen (in units of charge) as quantified by square wave voltammetry on the spent media from various cultures after 15 hours post MV (6 $\mu$M) treatment and incubation at 30 °C in continuous light (40 μmol⋅s-1⋅m-2). Individual traces and abiotic controls are shown in Figure S15. Error bars represent standard deviations from the mean for three biological replicates.

Cell growth and oxygen evolution measurements demonstrated that the toxic effects of MV were bypassed in mutant strains, and that this was not due to MV degradation. To investigate the mechanism of toxicity further, endpoint measurements of the intracellular ROS content across all strains following MV treatment were determined. All MV-adapted mutants exhibited a >6-fold depletion in intracellular ROS relative to their parental wild-type strain (Figure 3G). This suggests the MV resistance phenotype is characterised by a suppression of intracellular ROS production. This is consistent with the lower photosynthetic rates observed in MV-adapted mutants (Fig. 3E), which would limit both the reduction of MV by the photosynthetic electron transport chain, as well as its oxidation by oxygen. Leveraging MV’s permeability39 and ability to act as an electron mediator40, we conducted electrochemical experiments (chronoamperometry) to characterise the photosynthetic reduction of MV in both wild-type and MV-resistant strains (Figure S14). In the absence of MV, wild-type strains produced small photocurrents of approximately 0.2 μA, upon addition of 6 μM MV, wild-type strains initially generated large photocurrents (of more than 5 μA), consistent with MV mediating electrons between the photosynthetic electron transport chain and the electrode. These photocurrents rapidly decreased over time and the biofilm bleached, indicating cell toxicity. In contrast, MV-adapted strains maintained similar photocurrents in the absence of MV to those recorded in the presence of MV, and their biofilms remained green. The inability of MV to act as an electron mediator in MV-adapted mutants suggests these strains exhibit diminished intracellular reduction of MV. To test this, we performed square wave voltammetry to estimate the concentration of MV accumulated extracellularly during photoautotrophic growth following MV treatment. As shown in Figure 3H, after 15 hours all MV-resistant strains displayed approximately twice as much MV in their supernatants than their parental strains, confirming that resistance is achieved by decreasing intracellular accumulation of MV.

# Discussion

In this study, we demonstrated that multiple cultures from two separate wild-type strains of the model freshwater cyanobacterium *Synechocystis* could rapidly and reproducibly evolve spontaneous resistance to MV. Resistant strains emerged on average 10 days after MV treatment, which is equivalent to 20-30 generations41–43. By comparison, previous studies of *E. coli* have demonstrated that antibiotic resistant strains only emerge after more than 100 generations44. Despite wild-type cultures bleaching after treatment with MV at concentrations as low as 1 μM, emergence of viable cultures was observed during liquid cultivation after a period of 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 chemical degradation. Emergence of spontaneous mutants was observed consistently for both wild type *Synechocystis* strains, but occurred only when MV was added during mid-log growth and at concentrations lower than 100 μM. Additionally, we observed that emergence of MV-resistant mutants only occurred when MV was introduced during mid-log phase (Figure S4). Considering that genome ploidy levels are known to fluctuate during different growth phases, with *Synechocystis* cells containing significantly more genome copies in exponential phase than in linear or stationary phase10, these results hinted at a role of polyploidy for cyanobacterial adaptive mechanisms. Not only did we recapitulate the same mutations (R115G) observed in previous research33, but we expanded on their findings by identifying a novel histidine substitution in the same conserved R115 residue and by providing a comprehensive whole genome analysis and identifying the growth conditions in which such adaptive evolution under photoautotrophic cultivation is successful. 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 photosynthesis45,46.

The results indicated that resistance was due to genetic changes, and all resistant strains, with the exception of mvR09\_Howe, harboured non-synonymous mutations in ABC transporters (Table 1). This suggests that resistance may be achieved by altering methyl viologen membrane transport, which agrees with previous studies33–35,47 and, for the first time, was corroborated experimentally using electrochemical techniques (Figure 3H, Figure 15). Future work should focus on understanding how the observed mutations in MV-resistant lead to differential MV transport. These mutations might increase the rate of MV efflux by changing transporter substrate specificity48,49 or rate of ATP-dependent efflux50,51; or inhibit the activity of transporters which perform methyl viologen uptake52,53. It is also possible that these mutations do not affect the transport of methyl viologen directly, but instead affect the secretion of antioxidants or extracellular polymeric substances which may neutralise MV’s electrochemical activity53–55, and therefore its cytotoxicity. This argument may be favoured by the fact that the ABC transporter genes *hlyB* (mutated in mvR01, mvR02, mvR03) and *hlyD* (mutated in mvR06) both encode subunits of the Type I Secretory System of *Synechocystis,* which has been shown to export diverse compounds including antibiotics, acids, and the S-layer protein Sll1952 56,57. Mutations in Sll1952 (the main substrate of the HlyB translocation system56) have been observed in previous ALE studies of *Synechocystis* exposed to acid stress58. We tested whether the MV-resistant strains were resistant to additional treatments other than MV, including photosynthetic inhibitors and ROS-inducing treatments (Figure S16). However, we could not observe any significant differences in growth inhibition between wild-type and mvR mutants for the tested treatments. This would suggest that the observed mutations only affect the rate of MV transport or of MV-specific inhibitors. Finally, changes in the ATP/NADPH ratio resulting from alterations to transporter function could in turn have wider effects on photosynthetic metabolism59, perhaps accounting for changes observed in growth rate and oxygen evolution in the absence of MV (Figure 3A-B & E).

Genomic sequencing revealed a surprisingly high frequency of shared mutational events between independent MV-resistant strains. Adaptive evolution was performed in different flasks, inoculated from individual colonies and subjected to independent MV treatments, so it is unlikely that such shared mutational events were a result of cross-contamination or multiple homozygous subpopulation within each colony. A principal component analysis (Figure S9) further suggests that strains with shared mutational events differed significantly between each other when all mutations were considered, again indicating that cross-contamination had not occurred. Shared non-synonymous mutations were observed only in MV-adapted strains isolated from the same parental wild-type strain which already contained these alleles at low frequencies (Table 1). In addition, MV-adapted strains exhibited a global shift in frequencies of other alleles in comparison to their parental wild-type strains (Figure 2 C-D).

This is, to our knowledge, the first experimental evidence of rapid genetic adaptation in bacteria brought about by increases in frequencies of pre-existing naturally occurring chromosomal alleles conferring resistance to a selection agent. Consistent with this, similar adaptation in bacteria has been observed with multicopy plasmids or with genetically engineered strains with chromosomes modified such that different copies contain different antibiotic resistance cassettes60–62. Because MV resistant strains were isolated from clonal colonies, we are confident our results are wholly described by changes in allele frequencies rather than changes in the abundance of genetically distinct sub-populations. Future experiments could further characterise changes in allele frequencies through the application of single cell sequencing techniques63,64.

Our results suggest that polyploidy in bacteria facilitates rapid genetic adaptation to stressful conditions. As suggested by theoretical models1,61, bacterial polyploidy could provide an enhanced ability to explore the space of genotypes without disrupting genes with positive fitness effects (as with gene duplications). One outstanding question is why heterozygosity in polyploid bacteria exists under natural conditions, especially in the example presented here where the alleles enriched in MV-adapted strains are associated with a reduced fitness in the absence of MV (Figure 3A-B). It is possible that the MV-resistance alleles are neutral at low frequencies, and their presence at these frequencies is due to stochastic effects (genetic drift)65. Alternatively, their persistence may be due to balancing selection. On this model the MV-resistance alleles would be beneficial under some natural growth conditions, albeit deleterious under others. The variation in fitness might, for example, be dependent on time (e.g. growth phase) or location of cells in a culture. This temporal or spatial heterogeneity would result in a balancing selection and stable maintenance of the MV-resistant allele. This suggests that, as in plants66, balancing selection mechanisms may play an important part in the evolutionary diversity and adaptations of cyanobacteria and other polyploid prokaryotes by allowing for the maintenance of low-frequency alleles. Whilst the exact mechanisms of this balancing selection may differ between mutations, previous computational models and experimental data on multi-copy plasmids has demonstrated how fluctuating selection pressure can lead to the maintenance of heterozygosity over multiple generations1,61. Cyanobacteria are known to secrete and be exposed to toxins (including photosynthetic inhibitors67), and also to experience fluctuations in oxidative stress caused by daily and seasonal changes in light intensity, temperature, and nutrient availability14. It is therefore likely that such ecological and metabolic conditions may provide the selection for the maintenance of low-frequency genomic alleles associated with oxidative stress resistance, such as the mutations identified in this study. Polyploidy has been identified in ecological keystone cyanobacteria, including in the marine diazotroph *Trichodesmia* in field conditions8 and is widespread among both marine and freshwater picocyanobacteria68. Additionally, multiple studies have identified spatiotemporal changes in allele frequencies of *Microcystis* in freshwater ecosystems throughout an algal bloom season. Further ecological and metagenomic studies are required to determine if these changes in allele frequencies are occurring at a single cell or population level.

Genome polyploidy is an often discussed but rarely characterised phenomenon in bacteria, with important physiological and evolutionary consequences. Previous work has suggested polyploidy provides dosage-dependent gene regulation69, storage of phosphate70, or even facilitate DNA repair for double homologous recombination71. Taken together, the results presented here indicate that – in cyanobacteria as in eukaryotic clonal systems – polyploidy provides increased genetic diversity and can facilitate rapid adaptation to new selection pressures.

# STAR Methods

## Experimental model and study participant details

## Strains and maintenance of *Synechocystis* sp. PCC 6803

During this study two substrains of the model cyanobacterium *Synechocystis* sp. PCC 6803 were used, which were originally derived from the reference GT-Kazusa strain. The “Howe” labtype was maintained in the laboratory of Professor. Christopher J. Howe (University of Cambridge, UK). The “Nixon” type was a kind gift from Dr. Elinor Thompson (University of Greenwich) and was originally obtained from the laboratory of Peter J. Nixon (Imperial College London, U.K.). Cryopreserved stocks of cultures were streaked onto BG11 Petri dishes containing 1.5% of agar. Single colonies appeared after approximately 14 days following incubation at 30℃ under white light illumination (40 μmol⋅s-1⋅m-2). Liquid cultures were maintained in BG11, prepared according to the standard protocol described by Rippka et al. (1979)72, and kept well-mixed by shaking at 100 rpm.

## Adaptive laboratory evolution

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 25 cm2 tissue culture flasks with breathable lids (Nunc). Cell growth was monitored by quantifying the optical density at 750 nm with a UV-Vis Spectrophotometer (Shimadzu) using BG11 as a blank. To prevent cross-contamination, measurements were taken directly through the flasks instead of cuvettes. MV stock solution was prepared by dissolving methyl viologen dichloride hydrate powder (Sigma) in autoclaved deionised water at a stock concentration of 6 mM, prior to filter-sterilising using a 0.22 μm filter and storing at -20 ℃. For MV adaptation experiments, MV was added into culture at different growth phases. Where different concentrations of MV were added into cultures (Figure 1), 1000x stock solutions for each tested working concentrations were prepared so the same volume of liquid was added in all conditions. The same volume of sterilised water was added for negative control conditions containing no MV. After adapted cultures emerged 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 sequencing. The same cultures were also inoculated on BG11 + 6 μM MV and spotted on MV containing plates in parallel to confirm that the resistance was maintained following growth in the absence of MV.

Method details

## Genomic DNA purification and sequencing

For genomic DNA purification, *Synechocystis* cultures inoculated from single colonies were grown photoautotrophically (40 μmol⋅s-1⋅m-2) until an OD750 of 1, and then harvested at 5000 x g for 5 minutes. The volume of culture to pellet was calculated to obtain a total chlorophyll *a* mass of 40 μg, calculated according to the equation: [chl*a*] (mg/ml) = (OD680-OD750)x10.85473. The supernatant was then discarded, and the pellet was resuspended in 170 μL of freshly prepared Smoker B buffer, containing 50 mM Tris/HCl (pH 8), 50 mM EDTA, 1 % (v/v) Triton, X-100, 20 mg/mL lysozyme, supplemented with 30 μL of 12 mgmL-1 RNase solution. This pre-lysis mixture was incubated at 37 ℃ for 1 hour. Following this, the tubes were vigorously vortexed and supplemented with 20 μL of 20 mgmL-1 Proteinase K solution. Another round of incubation was then carried out at 56 ℃ for one hour. To homogenize the sample further, glass beads were added to the solution, which was then subjected to bead beating for 5 cycles of 1 min beating followed by 1 min on ice. After bead beating, the mixture was centrifuged at 5000 x g 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 ℃) EDTA-free elution buffer to the column, allowing it to sit for 3 minutes, and then centrifuging at 11000 x g for 1 minute. A subsequent addition of 50 μL of elution buffer was performed, followed by a 3-minute waiting period, and a final centrifugation to collect highly pure and concentrated genomic DNA. The concentration of purified gDNA was quantified spectrophotometrically (Nanodrop, ThermoFisher) and its integrity assessed by gel electrophoresis. Library preparation and genome sequencing was performed by Novogene (Cambridge, UK) using Illumina PE150 technology.

## Spectrophotometric quantification of intracellular ROS

Quantification of intracellular ROS was performed according to a protocol described previously74. Before incubation with DCF, *Synechocystis* cultures were grown in BG11 + 6 uM (light intensity= 40 μmol⋅s-1⋅m-2) starting from an initial OD750 = 0.5. After 15 hours, the OD750 of the cultures was determined spectrophotometrically and then 2 mL of each culture was harvested by centrifugation (5000 x g for 5 minutes). The pellets were resuspended in PBS buffer + 2.5 μM of DCF and, following incubation at room temperature for 1 hour, bulk fluorescence was quantified in 3 mL quartz glass cuvettes using a fluorimeter (JASCO) set at λex = 485 nm, λem = 525 nm, 5 ms response time, and “medium” sensitivity. For each sample incubated with DCF, a corresponding sample with the same culture and buffer but no DCF was used as blank.

## Oxygen electrode measurements

Photosynthetic oxygen evolution rates and respiration rates were determined with 2 mL cell cultures at a concentration of 4 nmol chlorophyll *a* 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 μmol⋅s-1⋅m-2) 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. For dark-adjustments, 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.

## Electrochemical characterisation

To quantify extracellular electron transfer activities of *Synechocystis* 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 (PalmSense). These measurements were conducted on custom made flat photoelectrochemical cells containing indium tin oxide-coated PET working electrodes and open-air platinum-coated carbon cloth counter electrodes. Measurements were performed under chopped light (100 μmol⋅s-1⋅m-2) and dark periods lasting two hours each. For each experiment, cultures containing a total of 250 nmol of chlorophyll *a* were pelleted, washed, and loaded on the flat ITO electrodes.

To quantify MV concentration In the supernatant, square wave voltammetries were performed using XX reference electrodes, XX working and XX counter electrodes. Measurements were performed at room temperature and in anoxic conditions (purging with nitrogen for XX minutes). Measurements were taken with a XX potentiostat (add make) with a scan rate of XX. Spent media were obtained by centrifugation of cultures for 5 min at 5000 x g. Before centrifugation, all cultures were treated with 6 μM MV for 15 hours at 30°C under continuous white light (40 μmol⋅s-1⋅m-2).

**Quantification and statistical analysis**

**Bioinformatic analysis**

The raw reads (forward and reverse) obtained from the WGS experiments were paired using the “Set Paired Reads” function in Geneious (with the “Illumina Read technology”, “Paired End”, “Expected Distance” = 150 as parameters). Regions with more than a 5% chance of an error per base were trimmed from the paired-end reads using the “Trim End” function in Geneious. Paired and trimmed reads were then aligned to various reference genomes using Bowtie 274 with the options “end to end alignment” and “high sensitivity”. Variant analysis was performed in Geneious using the “Variant Analysis” tool. To ensure that at any positions represented alleles and not sequencing errors the minimum variant frequency was set to 0.2, the maximum variant p-value was set to 10-6 (0.0001% to see variants by chance) and the minimum strand bias p-value was set to 10-6 when exceeding 65% of strand bias. The resulting variants were filtered, analysed and visualised using custom python scripts, which are publicly available at https://github.com/scaralbi/MV\_Resistance

**Additional resources**

Raw data (DNA sequencing reads) from whole genome sequencing experiments are publicly available at: <https://doi.org/10.17863/CAM.113897>

**Acknowledgements**

We thank Dr. Elinor Thompson for the gift of the wild-type (“Nixon”) *Synechocystis* strain, and Rachel Egan and James Bridson for technical assistance. This work was funded by the BBSRC (BB/M011194/1 to A.S. and J.M.L, BB/R011923/1 to J.Z., BB/X511092/1 to P.B.) and the EPSRC (EP/X525686/1 to P.B) J.M.L. received additional financial support from the Worshipful Company of Leathersellers and Trinity Hall, Cambridge. D.K. acknowledges funding from the Gates Cambridge Trust with support of the Benn W Levy Trust.

**Author contributions**

Conceptualization, A.S., J.M.L., and C.J.H.; methodology, A.S., J.M.L., P.B., D.K., J.Z. and C.J.H; software, A.S.; formal analysis, A.S., J.M.L., and C.J.H; investigation, A.S., J.M.L.; writing – original draft, A.S., J.M.L., and C.J.H; writing – review & editing, A.S., J.M.L., P.B., D.K., J.Z., C.J.H.; visualization, A.S.; supervision and funding acquisition, C.J.H..

**Declarations of interests**

The authors declare no competing interests.

**References**

1. Garoña, A., Santer, M., Hülter, N. F., Uecker, H. & Dagan, T. Segregational drift hinders the evolution of antibiotic resistance on polyploid replicons. *PLoS Genet* **19**, (2023).

2. Shi, A., Fan, F. & Broach, J. R. Microbial adaptive evolution. *J Ind Microbiol Biotechnol* **49**, 76 (2022).

3. Barrett, R. D. H. & Schluter, D. Adaptation from standing genetic variation. *Trends Ecol Evol* **23**, 38–44 (2008).

4. Springael, D. & Top, E. M. Horizontal gene transfer and microbial adaptation to xenobiotics: New types of mobile genetic elements and lessons from ecological studies. *Trends Microbiol* **12**, 53–58 (2004).

5. Lenski, R. E. Experimental evolution and the dynamics of adaptation and genome evolution in microbial populations. *The ISME Journal 2017 11:10* **11**, 2181–2194 (2017).

6. Adam, M., Murali, B., Glenn, N. O. & Potter, S. S. Epigenetic inheritance based evolution of antibiotic resistance in bacteria. *BMC Evol Biol* **8**, (2008).

7. Ionescu, D. *et al.* Heterozygous, Polyploid, Giant Bacterium, Achromatium, Possesses an Identical Functional Inventory Worldwide across Drastically Different Ecosystems. *Mol Biol Evol* **38**, 1040–1059 (2021).

8. Sargent, E. C. *et al.* Evidence for polyploidy in the globally important diazotroph Trichodesmium. *FEMS Microbiol Lett* **363**, 244 (2016).

9. Soppa, J. Non-equivalent genomes in polyploid prokaryotes. *Nature Microbiology* vol. 7 186–188 Preprint at https://doi.org/10.1038/s41564-021-01034-3 (2022).

10. Griese, M., Lange, C. & Soppa, J. Ploidy in cyanobacteria. *FEMS Microbiol Lett* **323**, 124–131 (2011).

11. Peschek, G. A., Bernroitner, M., Sari, S., Pairer, M. & Obinger, C. Life Implies Work: A Holistic Account of Our Microbial Biosphere Focussing on the Bioenergetic Processes of Cyanobacteria, the Ecologically Most Successful Organisms on Our Earth. *Bioenergetic Processes of Cyanobacteria* 3–70 (2011) doi:10.1007/978-94-007-0388-9\_1.

12. Lumian, J. *et al.* Biogeographic distribution of five Antarctic cyanobacteria using large-scale k-mer searching with sourmash branchwater. *Front Microbiol* **15**, 1328083 (2024).

13. Miller, S. R. & Castenholz, R. W. Evolution of thermotolerance in hot spring cyanobacteria of the genus Synechococcus. *Appl Environ Microbiol* **66**, 4222–4229 (2000).

14. Latifi, A., Ruiz, M. & Zhang, C. C. Oxidative stress in cyanobacteria. *FEMS Microbiology Reviews* vol. 33 258–278 Preprint at https://doi.org/10.1111/j.1574-6976.2008.00134.x (2009).

15. Weidel H & Russo M. Studien über das Pyridin [Studies of pyridine]. *Monatshefte für Chemie (in German)* **3**, 850–885 (1882).

16. Michaelis, L. & Hill, E. S. The viologen indicators. *J Gen Physiol* **16**, 859–873 (1933).

17. Brian, R. C., Homer, R. F., Stubbs, J. & Jones, R. L. *A new herbicide 1’-ethylene-2 : 2’-dipyridylium dibromide*. *Nature* **15** (1958).

18. Dong, F. *et al.* An engineered, non-diazotrophic cyanobacterium and its application in bioelectrochemical nitrogen fixation. *Cell Rep Phys Sci* **2**, (2021).

19. Milton, R. D. *et al.* Bioelectrochemical Haber-Bosch Process: An Ammonia-Producing H 2 /N 2 Fuel Cell . *Angewandte Chemie* **129**, 2724–2727 (2017).

20. Lawrence, J. M. *et al.* Synthetic Biology and Bioelectrochemical Tools for Electrogenetic System Engineering. *Sci. Adv* vol. 8 (2022).

21. Aulenta, F. *et al.* Electron transfer from a solid-state electrode assisted by methyl viologen sustains efficient microbial reductive dechlorination of TCE. *Environ Sci Technol* **41**, 2554–2559 (2007).

22. Jiang, Q. *et al.* Synthetic engineering of a new biocatalyst encapsulating [NiFe]-hydrogenases for enhanced hydrogen production. *J Mater Chem B* **11**, 2684–2692 (2023).

23. Gemünde, A., Lai, B., Pause, L., Krömer, J. & Holtmann, D. Redox Mediators in Microbial Electrochemical Systems. *ChemElectroChem* vol. 9 Preprint at https://doi.org/10.1002/celc.202200216 (2022).

24. Hance, R. J., Byast, T. H. & Smith, P. D. Apparent decomposition of paraquat in soil. *Soil Biol Biochem* **12**, 447–448 (1980).

25. Sétif, P. Electron-transfer kinetics in cyanobacterial cells: Methyl viologen is a poor inhibitor of linear electron flow. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1847**, 212–222 (2015).

26. Bus, J. S., Aust, S. D. & Gibson, J. E. Superoxide-and singlet oxygen-catalyzed lipid peroxidation as a possible mechanism for paraquat (methyl viologen) toxicity. *Biochem Biophys Res Commun* **58**, (1974).

27. Thomas, D. J., Avenson, T. J., Thomas, J. B. & Herbert, S. K. A Cyanobacterium Lacking Iron Superoxide Dismutase Is  Sensitized to Oxidative Stress Induced with Methyl Viologen but Is  Not Sensitized to Oxidative Stress Induced with  Norflurazon. *Plant Physiol* **116**, 1593 (1998).

28. Campbell, W. S. & Laudenbach, D. E. Characterization of four superoxide dismutase genes from a filamentous cyanobacterium. *J Bacteriol* **177**, 964–972 (1995).

29. Li, T. *et al.* Differential expression and localization of Mn and Fe superoxide dismutases in the heterocystous cyanobacterium *Anabaena* sp. Strain PCC 7120. *J Bacteriol* **184**, 5096–5103 (2002).

30. Kim, J. H. & Suh, K. H. Light-dependent expression of superoxide dismutase from cyanobacterium Synechocystis sp. strain PCC 6803. *Arch Microbiol* **183**, 218–223 (2005).

31. Mata-Cabana, A., García-Domínguez, M., Florencio, F. J. & Lindahl, M. Thiol-Based Redox Modulation of a Cyanobacterial Eukaryotic-Type Serine/Threonine Kinase Required for Oxidative Stress Tolerance. *https://home.liebertpub.com/ars* **17**, 521–533 (2012).

32. Gao, H. & Xu, dong. The cyanobacterial NAD kinase gene sll1415 is required for photoheterotrophic growth and cellular redox homeostasis in *Synechocystis* sp. strain PCC 6803. *J Bacteriol* **194**, 218–224 (2012).

33. Prosecka, J. *et al.* A novel ATP-binding cassette transporter is responsible for resistance to viologen herbicides in the cyanobacterium *Synechocystis* sp. PCC 6803. *FEBS Journal* **276**, 4001–4011 (2009).

34. Nefedova, L. N., Fantin, Y. S., Zinchenko, V. V. & Babykin, M. M. The *prqA* and *mvrA* Genes Encoding Drug Efflux Proteins Control Resistance to Methyl Viologen in the Cyanobacterium *Synechocystis* sp. PCC 6803. *Russ J Genet* **39**, 264–268 (2003).

35. Babykin, M. M. *et al.* On the involvement of the regulatory gene *prqR* in the development of resistance to methyl viologen in cyanobacterium *Synechocystis* sp. PCC 6803. *Russ J Genet* **39**, 18–24 (2003).

36. Oh, S. & Montgomery, B. L. Roles of CpcF and CpcG1 in Peroxiredoxin-Mediated Oxidative Stress Responses and Cellular Fitness in the Cyanobacterium *Synechocystis* sp. PCC 6803. *Front Microbiol* **10**, (2019).

37. von Berlepsch, S. *et al.* The acyl-acyl carrier protein synthetase from *Synechocystis* sp. PCC 6803 mediates fatty acid import. *Plant Physiol* **159**, 606–617 (2012).

38. Mustila, H., Allahverdiyeva, Y., Isojärvi, J., Aro, E. M. & Eisenhut, M. The bacterial-type [4Fe-4S] ferredoxin 7 has a regulatory function under photooxidative stress conditions in the cyanobacterium *Synechocystis* sp. PCC 6803. *Biochim Biophys Acta* **1837**, 1293–1304 (2014).

39. Jones, R. W., Gray, T. A. & Garland, P. B. A Study of the Permeability of the Cytoplasmic Membrane of *Escherichia coli* to Reduced and Oxidized Benzyl Viologen and Methyl Viologen Cations: Complications in the Use of Viologens as Redox Mediators for Membrane-Bound Enzymes. *Biochem Soc Trans* **4**, 671–673 (1976).

40. Dong, F., Lee, Y. S., Gaffney, E. M., Liou, W. & Minteer, S. D. Engineering Cyanobacterium with Transmembrane Electron Transfer Ability for Bioelectrochemical Nitrogen Fixation. *ACS Catal* **11**, 13169–13179 (2021).

41. Foster, J. S., Singh, A. K., Rothschild, L. J. & Sherman, L. A. Growth-phase dependent differential gene expression in Synechocystis sp. strain PCC 6803 and regulation by a group 2 sigma factor. *Arch Microbiol* **187**, 265–279 (2007).

42. Vermaas, W. F. J., Rutherfordt, A. W. & Hanssontt, O. *Site-Directed Mutagenesis in Photosystem II of the Cyanobacterium Synechocystis Sp. PCC 6803: Donor D Is a Tyrosine Residue in the D2 Protein (Photosynthesis/Protein Engineering/Oxygen Evolution/Electron Paramagnetic Resonance/Electron Transport)*. *Proc. Natl. Acad. Sci. USA* vol. 85 (1988).

43. Liu, X. & Curtiss, R. Nickel-inducible lysis system in *Synechocystis* sp. PCC 6803. *Proc Natl Acad Sci U S A* **106**, 21550–21554 (2009).

44. Xing, Y., Kang, X., Zhang, S. & Men, Y. Specific phenotypic, genomic, and fitness evolutionary trajectories toward streptomycin resistance induced by pesticide co-stressors in Escherichia coli. *ISME Communications 2021 1:1* **1**, 1–11 (2021).

45. Dann, M. *et al.* Enhancing photosynthesis at high light levels by adaptive laboratory evolution. *Nat Plants* **7**, 681–695 (2021).

46. Sun, H. *et al.* Engineered hypermutation adapts cyanobacterial photosynthesis to combined high light and high temperature stress. *Nat Commun* **14**, (2023).

47. Ongley, S. E., Pengelly, J. J. L. & Neilan, B. A. A multidrug efflux response to methyl viologen and acriflavine toxicity in the cyanobacterium *Synechocystis* sp. PCC6803. *J Appl Phycol* **28**, 2793–2803 (2016).

48. Srikant, S., Gaudet, R. & Murray, A. W. Selecting for Altered Substrate Specificity Reveals the Evolutionary Flexibility of ATP-Binding Cassette Transporters. *Current Biology* **30**, 1689-1702.e6 (2020).

49. Maqbool, A. *et al.* The substrate-binding protein in bacterial ABC transporters: dissecting roles in the evolution of substrate specificity. *Biochem Soc Trans* **43**, 1011–1017 (2015).

50. Prieß, M., Göddeke, H., Groenhof, G. & Schäfer, L. V. Molecular Mechanism of ATP Hydrolysis in an ABC Transporter. *ACS Cent Sci* **4**, 1334–1343 (2018).

51. Davidson, A. L. Mechanism of coupling of transport to hydrolysis in bacterial ATP-binding cassette transporters. *J Bacteriol* **184**, 1225–1233 (2002).

52. Xi, J., Xu, P. & Xiang, C. Bin. Loss of AtPDR11, a plasma membrane-localized ABC transporter, confers paraquat tolerance in *Arabidopsis thaliana*. *The Plant Journal* **69**, 782–791 (2012).

53. Fujita, M. & Shinozaki, K. Identification of Polyamine Transporters in Plants: Paraquat Transport Provides Crucial Clues. *Plant Cell Physiol* **55**, 855–861 (2014).

54. Fujita, M. *et al.* Natural variation in a polyamine transporter determines paraquat tolerance in Arabidopsis. *Proc Natl Acad Sci U S A* **109**, 6343–6347 (2012).

55. Hu, X., Luo, K., Ji, K., Wang, L. & Chen, W. ABC transporter *slr0982* affects response of Synechocystis sp. PCC 6803 to oxidative stress caused by methyl viologen. *Res Microbiol* **173**, 103888 (2022).

56. Agarwal, R., Whitelegge, J. P., Saini, S. & Shrivastav, A. P. The S-layer biogenesis system of Synechocystis 6803: Role of Sll1180 and Sll1181 (E. coli HlyB and HlyD analogs) as type-I secretion components for Sll1951 export. *Biochim Biophys Acta Biomembr* **1860**, 1436–1446 (2018).

57. Uchiyama, J. *et al.* Characterization of ABC transporter genes, sll1180, sll1181, and slr1270, involved in acid stress tolerance of *Synechocystis* sp. PCC 6803. *Photosynth Res* **139**, 325–335 (2019).

58. Uchiyama, J. *et al.* Genomic analysis of parallel-evolved cyanobacterium *Synechocystis* sp. PCC 6803 under acid stress. *Photosynth Res* **125**, 243–254 (2015).

59. Kugler, A. & Stensjö, K. Optimal energy and redox metabolism in the cyanobacterium Synechocystis sp. PCC 6803. *npj Systems Biology and Applications 2023 9:1* **9**, 1–13 (2023).

60. Garoña, A., Hülter, N. F., Picazo, D. R. & Dagan, T. Segregational Drift Constrains the Evolutionary Rate of Prokaryotic Plasmids. *Mol Biol Evol* **38**, 5610–5624 (2021).

61. Santer, M., Kupczok, A., Dagan, T. & Uecker, H. Fixation dynamics of beneficial alleles in prokaryotic polyploid chromosomes and plasmids. *Genetics* **222**, (2022).

62. Rodriguez-Beltran, J. *et al.* Multicopy plasmids allow bacteria to escape from fitness trade-offs during evolutionary innovation. *Nat Ecol Evol* **2**, 873–881 (2018).

63. Wang, B. *et al.* Single-cell massively-parallel multiplexed microbial sequencing (M3-seq) identifies rare bacterial populations and profiles phage infection. *Nature Microbiology 2023 8:10* **8**, 1846–1862 (2023).

64. Poirion, O., Zhu, X., Ching, T. & Garmire, L. X. Using single nucleotide variations in single-cell RNA-seq to identify subpopulations and genotype-phenotype linkage. *Nat Commun* **9**, (2018).

65. Kimura, M. On the probability of fixation of mutant genes in a population. *Genetics* **47**, (1962).

66. Delph, L. F. & Kelly, J. K. On the importance of balancing selection in plants. *New Phytologist* **201**, 45–56 (2014).

67. Kaebernick, M. & Neilan, B. A. Ecological and molecular investigations of cyanotoxin production. *FEMS Microbiol Ecol* **35**, 1–9 (2001).

68. Weissenbach, J. *et al.* Ploidy levels in diverse picocyanobacteria from the Baltic Sea. *Environ Microbiol Rep* **16**, e70005 (2024).

69. Ohbayashi, R. *et al.* Coordination of Polyploid Chromosome Replication with Cell Size and Growth in a Cyanobacterium. *mBio* **10**, (2019).

70. Brück, P., Wasser, D. & Soppa, J. One Advantage of Being Polyploid: Prokaryotes of Various Phylogenetic Groups Can Grow in the Absence of an Environmental Phosphate Source at the Expense of Their High Genome Copy Numbers. *Microorganisms* **11**, (2023).

71. Van De Peer, Y., Mizrachi, E. & Marchal, K. The evolutionary significance of polyploidy. *Nature Reviews Genetics* vol. 18 411–424 (2017).

72. Rippka, R., Deruelles, J. & Waterbury, J. B. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J Gen Microbiol* **111**, 1–61 (1979).

73. Lea-Smith, D. J. *et al.* Thylakoid terminal oxidases are essential for the cyanobacterium *Synechocystis* sp. PCC 6803 to survive rapidly changing light intensities. *Plant Physiol* **162**, 484–495 (2013).

74. Rajneesh, Pathak, J., Chatterjee, A., Singh, S. P. & Sinha, R. P. Detection of Reactive Oxygen Species (ROS) in Cyanobacteria Using the Oxidant-sensing Probe 2’,7’-Dichlorodihydrofluorescein Diacetate (DCFH-DA). *Bio Protoc* **7**, (2017).