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Glutathione Facilitates Antibiotic Resistance and Photosystem I Stability during Exposure to Gentamicin in Cyanobacteria[▽]

Jeffrey C. Cameron and Himadri B. Pakrasi*

Department of Biology, Washington University, St. Louis, Missouri 63130

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Understanding mechanisms of antibiotic resistance is important to the fields of biology and medicine. We find that glutathione contributes to antibiotic resistance in the cyanobacterium *Synechocystis* sp. PCC 6803. Our results also suggest that glutathione protects photosystem I from oxidative damage resulting from growth in the presence of gentamicin.

Antibiotics target a broad array of cellular metabolic functions to inhibit growth or cause death. Bactericidal antibiotics primarily target cellular integrity, DNA replication, and translation (11). It has been demonstrated that reactive oxygen species (ROS) produced following antibiotic treatment lead to cell death (9, 12, 13). Additionally, antibiotics, such as the aminoglycoside gentamicin (Gm), may be directly involved in production of ROS (16). Antibiotic resistance can be manifested in many ways by the cell through enzymatic antibiotic modification, exclusion, export, and modulation of core metabolic pathways (4, 22).

Due to the formation of ROS following exposure to bactericidal antibiotics, the cellular antioxidant network likely plays a critical role in antibiotic resistance. Glutathione (GSH), a redox-active cellular thiol compound, may modulate antibiotic resistance. We recently found that glutathione is essential in the cyanobacterium *Synechocystis* sp. PCC 6803 (*Synechocystis* 6803) for acclimation to various cellular perturbations eliciting oxidative stress (5). In this study, we report that glutathione is important for protection from Gm toxicity in *Synechocystis* 6803. Further, our results demonstrate that photosystem I (PSI) levels are severely reduced in the Δ*gshB* mutant following application of Gm.

We utilized wild-type (WT) *Synechocystis* 6803 cells to test whether glutathione is involved in protection from application of Gm. We found that growth in liquid BG11 (1) medium is quickly inhibited in such cells following application of Gm at various concentrations (Fig. 1A). We also measured cellular GSH (Fig. 1B) and the GSH precursor γ-glutamylcysteine (γ-EC) (Fig. 1C) content by high-performance liquid chromatography (HPLC) as described previously (5) after 11 h of gentamicin treatment. Evidently, glutathione biosynthesis is stimulated in WT *Synechocystis* 6803 cells following the addition of Gm, suggesting a role in cellular protection from this antibiotic.

To further test the role of glutathione in protection from Gm, we utilized a glutathione synthetase (Δ*gshB*) mutant that we have previously characterized (5). The Δ*gshB* strain does not contain glutathione but instead accumulates γ-EC. We noticed that the Δ*gshB* mutant is extremely sensitive to growth in liquid media in the presence of Gm, despite the fact that the *gshB* gene was replaced with a Gm resistance cassette (Gm^r) in this strain (5). To further examine this phenotype, we compared growth of the Δ*gshB* and Δ*gshB*/T2086 strains in the presence of Gm (5 μg/ml) (Fig. 2). The Δ*gshB*/T2086 strain was constructed by fusing the *gshB* coding region to the *psbA2* promoter and expressing it at a second site in the genome of the Δ*gshB* mutant (5). While both strains contain a Gm^r cassette replacing the native *gshB* gene, the Δ*gshB*/T2086 strain is able to produce GSH. In the presence of Gm, the Δ*gshB* strain did not grow, while the Δ*gshB*/T2086 strain could (Fig. 2). Furthermore, using the broth dilution method and an initial cell concentration of approximately 9×10^7 cells/ml, the MIC for Gm (μg/ml) was determined to be 5 and 7.5 for the Δ*gshB* and Δ*gshB*/T2086 strains, respectively. The MIC for WT *Synechocystis* 6803, which does not contain a Gm^r gene, was 0.5 μg/ml. We also utilized a control strain, HT47-Gm, which harbors a Gm^r gene in addition to a histidine tag on the CP47 gene of PSII (18), and found the MIC to be 7.5 μg/ml. These data suggest that GSH and the Gm^r cassette are essential for Gm resistance in *Synechocystis* 6803.

Following application of Gm to Δ*gshB* liquid cultures, we noticed that the cultures turned a bluish color compared to that of the WT. To further investigate this phenomenon, we analyzed the cellular pigment composition in WT cells and in the Δ*gshB* strain grown in the absence or presence of Gm (5 μg/ml) using whole-cell absorption spectra as described previously (3) (Table 1). As a majority of the chlorophyll (Chl) in cyanobacteria is found in PSI, we also quantified the amount of P700 reaction centers by chemical difference spectroscopy (potassium ferricyanide oxidized, ascorbate reduced) using isolated membranes at a chlorophyll *a* concentration of 15 to 25 μg/ml as described previously (14). We found similar Chl:P700 ratios in the WT and Δ*gshB* strains during growth in the absence of Gm. However, in the presence of Gm, a significant increase in the Chl:P700 ratio was observed in the Δ*gshB* strain, suggesting a depletion of PSI (Table 1).

Next, we analyzed the content of PSI and photosystem II (PSII) reaction center proteins in isolated membranes. Proteins were separated by SDS-PAGE on 16% polyacrylamide gels and then transferred to nitrocellulose membranes using a semidry transfer apparatus (Bio-Rad, Hercules, CA). Figure 3

* Corresponding author. Mailing address: Department of Biology, Washington University, St. Louis, MO 63130. Phone: (314) 935-6853. Fax: (314) 935-6803. E-mail: pakrasi@biology2.wustl.edu.
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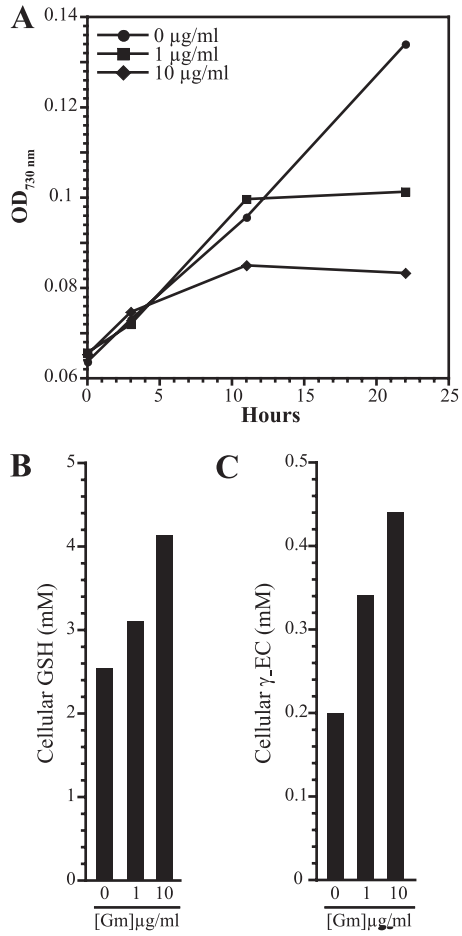

FIG. 1. Growth and cellular thiol concentrations of WT *Synechocystis* 6803 following application of gentamicin at various concentrations. Growth (A) was monitored as turbidity at 730 nm for 24 h. The cellular GSH (B) and γ-EC (C) concentrations were measured by HPLC after 11 h of growth in the presence of gentamicin.

shows immunoblots of the WT and Δ*gshB* samples probed with antibodies raised against the PSI and PSII reaction center proteins, PsaA/B and D1, respectively. The intensities of the cross-reacting bands were also quantified by densitometry using ImageJ software (Fig. 3B). We determined that the PsaA/B proteins were significantly reduced in the Δ*gshB* strain grown in the presence of Gm. However, the levels of D1 did not decrease following Gm treatment.

TABLE 1. Pigment content of *Synechocystis* 6803 strains[a]

Pigment	Mean (±SE) pigment content		
	WT	Δ*gshB* strain	Δ*gshB* strain + Gm
Chl-*a* (pg/cell)[b]	0.063 ± 0.0018	0.058 ± 0.0021	0.042 ± 0.0011
PC (pg/cell)[b]	0.45 ± 0.017	0.44 ± 0.018	0.35 ± 0.021
PC:Chl-*a* (pg/cell)[b]	7.18 ± 0.22	7.65 ± 0.18	8.42 ± 0.52
Chl-*a*:P700 (mol/mol)[c]	136.9 ± 6.9	132.8 ± 4.6	177.1 ± 10.3

[a] Values represent means ± standard errors from at least three independent experiments. Chl-*a*, chlorophyll *a*; PC, phycocyanin; Gm (5 μg/ml), gentamicin.
[b] Values obtained from whole-cell absorption spectra.
[c] Values calculated from chemical difference spectroscopy.

The results presented here show that glutathione is a critical player in Gm resistance mechanisms in *Synechocystis* 6803. Furthermore, GSH is required for resistance even when cells contain a functional Gm^r cassette. Our results also show that PSI, but not PSII, content is severely reduced in the Δ*gshB* strain following application of Gm.

Glutathione is a multifunctional molecule that is involved in core metabolic pathways, detoxification of xenobiotics, and maintenance of cellular redox poise. Many of these properties may come into play when the cell is challenged with an antibiotic. Glutathione *S*-transferases catalyze the addition of glutathione to xenobiotics to reduce toxicity and facilitate export (2). Further, GSH could also function in moderating the toxicity of ROS generated as a by-product of antibiotic toxicity. GSH and related thiols have also been suggested to modulate antibiotic sensitivity in nonphotosynthetic organisms (17, 20, 21). As glutathione biosynthesis likely evolved in ancestors of cyanobacteria during the evolution of oxygenic photosynthesis and was then transferred to many other organisms through lateral gene transfer (8), it is interesting to consider the role of

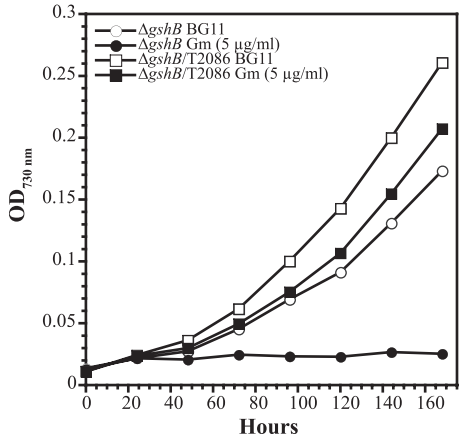

FIG. 2. Growth of the Δ*gshB* and complemented Δ*gshB*/T2086 strains in the presence of gentamicin (5 μg/ml).

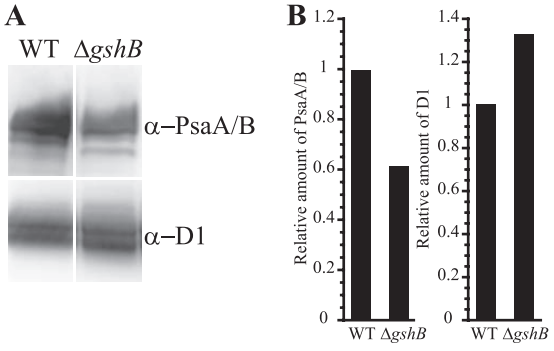

FIG. 3. Immunoblot analysis of PSI (PsaA/B) and PSII (D1) core proteins. (A) Isolated membranes from the WT or Δ*gshB* strain were loaded based on equal chlorophyll (2.5 μg Chl/lane). The Δ*gshB* strain was grown in the presence of Gm (5 μg/ml) prior to harvesting. (B) Immunoblot band intensity was quantified densitometrically using ImageJ software.

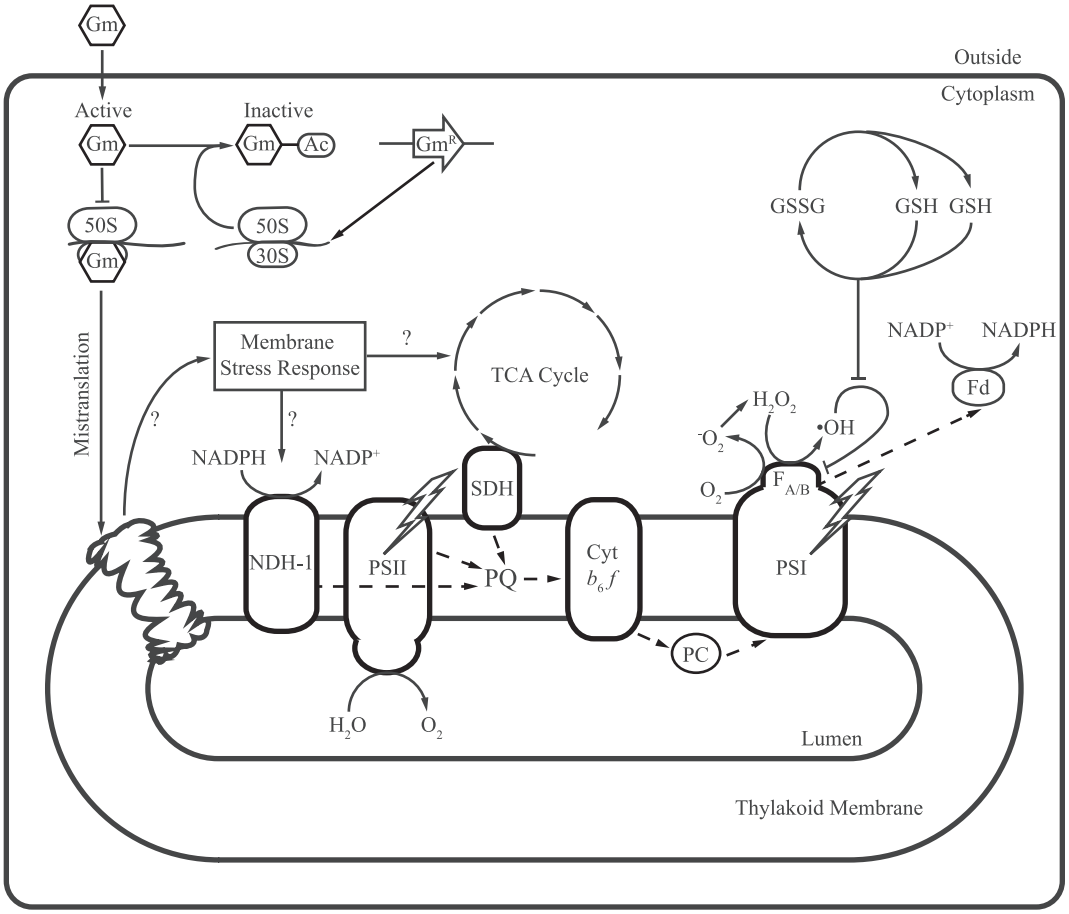

FIG. 4. Schematic diagram illustrating the role of glutathione in gentamicin resistance. Once gentamicin has entered the cell, it can be acetylated and detoxified by the product of the Gm^r gene or bind to the 30S subunit of the ribosome and cause mistranslation of proteins. Mistranslated proteins stimulate the membrane stress response, resulting in alteration of respiration and TCA cycle components. In cyanobacteria, the TCA cycle is incomplete. Stimulation of NADPH oxidation results in increased flow through the photosynthetic and respiratory electron transfer pathways. The terminal iron-sulfur clusters of photosystem I (PSI; $F_{A/B}$) can mediate Fenton chemistry and generation of the reactive hydroxyl radical (·OH). Glutathione (GSH) participates in ROS metabolism and can become oxidized to glutathione disulfide (GSSG).

cyanobacteria in modulating antibiotic resistance mechanisms across kingdoms.

Recently it has been shown that diverse classes of antibiotics result in the formation of ROS in Gram-positive and Gram-negative bacteria despite the differences in primary targets (9, 12, 13). Kohanski et al. (13) demonstrate that aminoglycoside antibiotics, such as kanamycin and gentamicin, stimulate oxidation of NADPH that is generated by the tricarboxylic acid (TCA) cycle. The resulting increase in respiratory activity generates superoxide ($^{-}O_2$) that is converted into H_2O_2 by superoxide dismutase. Superoxide and H_2O_2 are able to react with and liberate enzyme-bound iron-sulfur and promote Fenton reactions that result in the formation of the highly reactive hydroxyl radical (·OH). It has been suggested that iron-mediated Fenton reactions play a role in the cell-killing ability of many bactericidal antibiotics (9–13, 16). In fact, it was shown that alterations in import of iron or disruption of iron-sulfur cluster biogenesis could reduce the killing efficiency of aminoglycoside antibiotics (13). Therefore, increased cellular iron content could play a significant role in susceptibility to antibiotic-induced death.

Compared to heterotrophic bacteria, cyanobacteria contain at least an order of magnitude more iron within their cells (19) in order to supply the components of the photosynthetic and respiratory electron transfer chains, which reside in the same internal membrane system (7). PSI, the terminal component of the photosynthetic electron transfer chain, contains multiple iron-sulfur clusters that participate in electron transfer reactions (6). Therefore, PSI could be contributing to the ROS production following Gm application (Fig. 4). Our results suggest that glutathione plays a critical role in facilitating the removal of ROS and in the protection of PSI from oxidative damage. Depletion of PSI could be a consequence of ROS production and removal of iron-sulfur clusters, or it could represent an acclimation mechanism to increased ROS in a similar way that cells reduce PSI content following high-light treatment (15).

While there are striking differences in the metabolism of cyanobacteria and heterotrophic bacteria, a common thread is the necessity to ameliorate potential damage by ROS. Ancestors of modern-day cyanobacteria developed robust antioxidant networks and redox buffering systems as a necessity due to

the generation of molecular oxygen within the cell as a by-product of photosynthesis. These same mechanisms have been transferred to other bacteria and eukaryotes and have been elaborated upon to facilitate many aspects of cellular metabolism. Glutathione is a multifunctional molecule that is used by a plethora of organisms to prevent oxidative damage and also for detoxification of hazardous compounds. Therefore, it is likely that glutathione could play a role in resistance from multiple antibiotics in diverse organisms.

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