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Use of flow cytometry for efficient isolation of cyanobacterial mutants deficient in modulation of pigment level

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The modulation of pigment level is an essential adaptation response that allows photosynthetic organisms to adjust light harvesting to ambient conditions and to avoid oxidative damage caused by excess absorbed light (1,2). Cyanobacteria possess a macromolecular pigment complex termed phycobilisome, which harvests light energy and transfers it to the photosynthetic reaction center (3). This ultra-structure, which may comprise up to 50% of the soluble cellular protein, contains various apoprotein subunits, several different chromophors, as well as nonpigmented linkers required for assembly and energy transfer (3). Evaluation of the molecular mechanisms underlying degradation of the phycobilisome under stress conditions employs mutants that, unlike wild-type cells, do not degrade their pigments during nitrogen and sulfur deprivation. Such mutants have been thus far isolated by a visual screen (4–7). Several drawbacks are inherent to this method, and it is also laborious and time-consuming.

The visual screen is based on the observation that sulfur or nitrogen starvation triggers complete degradation of the phycobilisome (8). This results in a yellowish (bleached) color rather than the typical blue-green appearance of the cyanobacteria. To screen for mutants, mutagenized cells were plated on growth medium lacking added sulfur that was solidified with washed agar. Colonies growing on such medium, unless plated at a high density, were not starved enough to exhibit substantial pigment degradation. Therefore, this procedure resulted in very small and dense colonies that required screening under binoculars and several rounds of restreaking to obtain a clone exhibiting the desired phenotype. Furthermore, the rate of pigment degradation is affected by light intensity, and thus a color gradient appears in a single colony. This often leads to the isolation of false positives and generally makes it very difficult to obtain mutants exhibiting partial phenotypes; namely, those that degrade their pigments but to a lesser extent or at a slower rate compared to the wild-type strain.

Here we describe the successful application of a fluorescence-activated cell sorter (FACS®; BD Biosciences, San Jose, CA, USA) for the efficient isolation of mutants impaired in pigment degradation (nonbleaching mutants). The benefits of this technique can be amply demonstrated using the wild-type and the previously isolated nonbleaching mutant, nblRΩ. We analyzed the fluorescence properties of cells grown in complete and nutrient-depleted growth medium (Figure 1A). Fluorescence was measured following excitation at 590 nm, a wavelength that is efficiently absorbed by the cyanobacterial light-harvesting complex. A broad fluorescence peak (610–700 nm) is observed in cells grown in complete medium. Starved wild-type cells, on the other hand, exhibited lower fluorescence at this wavelength range, in agreement with the reduction of the major light-harvesting pigment during nutrient depletion (Figure 1A). The starvation of nblRΩ resulted in a somewhat decreased fluorescence. However, the fluorescence level of starved mutant culture is considerably higher than that of wild-type. It appeared to us that these clear differences in fluorescence could be utilized to enrich nonbleaching mutants from a mutagenized cell population. Excitation wavelength (633 nm) and emission conditions (band-pass filter, 660/20 nm) were chosen to achieve optimal separation between the populations of wild-type and nblRΩ cultures; only a minor fraction of the cells was found in the region of overlapping fluorescence (Figure 1B). Furthermore, the slower rate of phycobilisome degradation in sulfur, as compared to nitrogen-deprived cultures (R. Schwarz and A.R. Grossman, unpublished data), is reflected in elevated fluorescence exhibited by cultures starved for 16 h (Figure 1, A and B). Longer starvation periods (e.g., 48 h) mask the kinetics difference and result in apparently similar cultures (Figure 1C).

Taken together, these data support the idea that flow cytometry is appropriate for measuring quantitative differences in cellular pigments and encouraged us to employ FACS for the isolation of novel mutants impaired in the modulation of pigment level.

Random mutagenesis of *Synechococcus* sp. strain PCC 7942 was performed by transformation with a transposon-based inactivation library as previously described (9). Following the selection of transformants in the presence of 25 μg/mL kanamycin for 24 h, the cultures were washed once with growth medium lacking both nitrogen and sulfur, transferred to nitrogen- or sulfur-free medium, and sorted by FACS, following 16 h of starvation. The fluorescence characteristics of the mutagenized cell population appeared similar to those of wild-type (see Figure 1B) because only a small fraction of the cells was impaired in pigment degradation. Cells exhibiting fluorescence above a defined level (indicated by arrows in Figure 1B) were collected and plated, and colonies were restreaked onto complete media, nitrogen-free media, and sulfur-free media. The frequency of nonbleaching mutants in the sorted population was 5%–10%. Thus, using this new technique, we obtained over a hundred nonbleaching mutants within a very short time and with minimal effort. The visual screen commonly used today requires tens of thousands of colonies to be manually screened under binoculars and several rounds of restreaking to isolate several mutants.

Figure 1, C and D, shows cultures and absorbance spectra of wild-type, the previously isolated nonbleaching mutant, nblRΩ, and two of the newly isolated nonbleaching mutants, M56 and M104. The latter two mutants were characterized by PCR using primers to previously identified genes required for pigment degradation (data not shown). M56 was found to be impaired in a known gene, *nblA*; the aberrant gene produced a larger PCR product compared to that obtained from wild-type because the mutants were raised by insertional inactivation. This mutant has a substantial nonbleaching phenotype (Figure 1C). It retains most of its pigments during starvation as indicated by the absorbance peak at 620 nm (see Figure 1D; Reference 4). M104, on the other hand, degrades its phycobilisomes but to a lesser extent as compared to wild-type cells. This phenotype, which is a result of the inactivation of a novel gene required for pigment degradation (E. Sendersky, R. Lahmi, J. Shaltiel, A. Perelman, and R. Schwartz; manuscript in preparation), is more apparent during sulfur starvation (Figure 1, C and D).

Taken together, the newly described technique significantly promotes isolation of nonbleaching mutants, is rapid, efficient, and allows for the isolation of mutants that partially reduce their pigment level. Phenotypes of this kind are difficult to obtain by visual screen. In fact, all known components of the degradation pathway were identified by the aid of mutants exhibiting a substantial nonbleaching phenotype (4–7). Furthermore, the enrichment of mutants using FACS is potentially useful for a broader range of applications. For example, because induction of *nblA* by a foreign promoter is sufficient to cause phycobilisome degradation, FACS can be engaged for a "promoter hunt" by screening and sorting a cell population in which a promoterless *nblA* has been randomly inserted. Moreover, flow cytometry has been used for the characterization of phytoplankton composition based on fluorescence characteristics (10). Information gleaned from these studies may help to define the sorting conditions appropriate for the isolation of mutants differing in composition of various pigments in a broad range of organisms. For example, cyanobacterial mutants having aberrant levels of chlorophyll *a* may be isolated by sorting out cells exhibiting atypical fluorescence at 680 nm or longer wavelengths, follow-

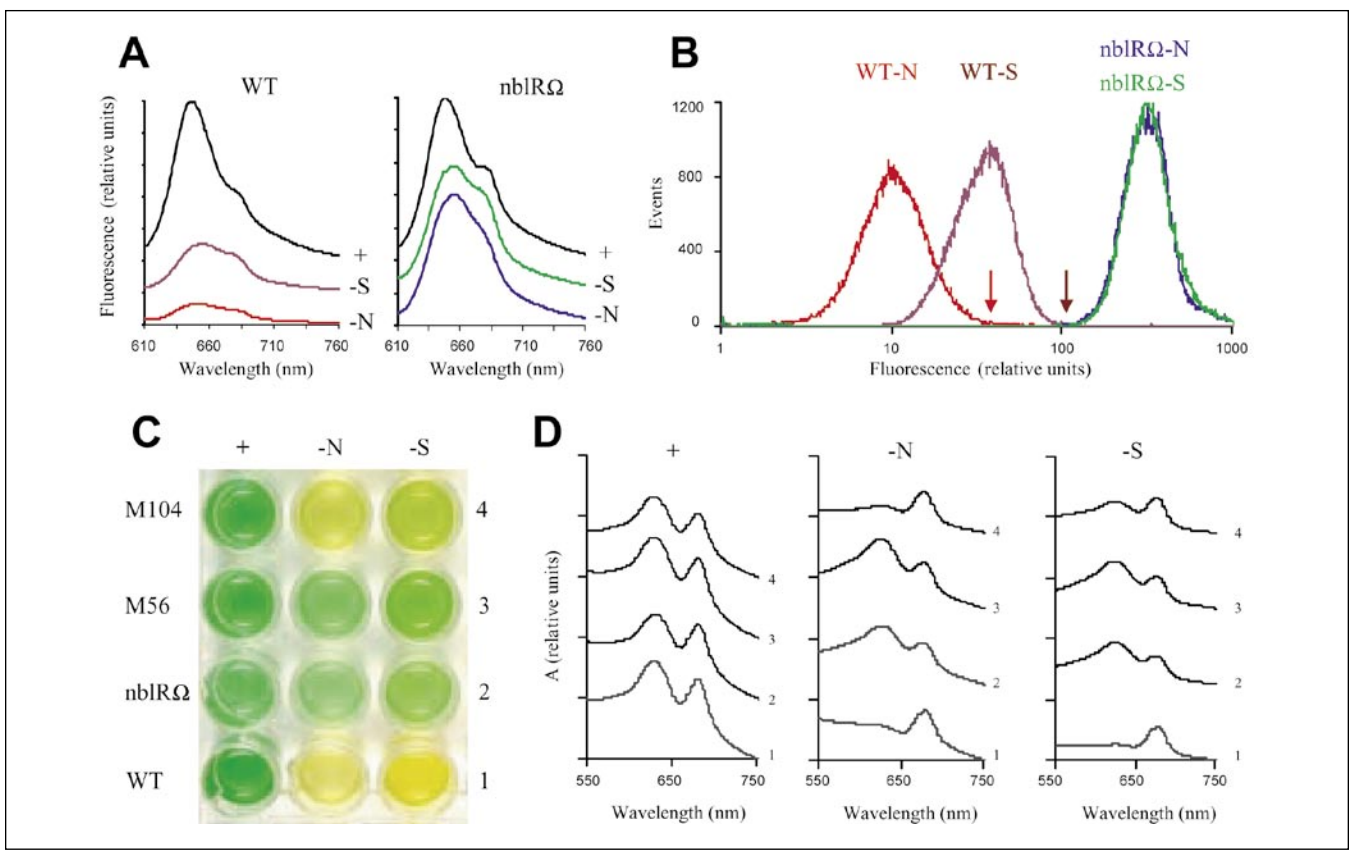

Figure 1. Spectral characteristics and photographs of *Synechococcus* sp. strain PCC 7942 and its nonbleaching mutants. (A) Fluorescence spectra and (B) cell counts of wild-type (WT) and nblR mutant (nblRΩ) grown in complete media (+) or starved for sulfur (-S) or nitrogen (-N) for 16 h. (A) Fluorescence was measured in a luminescence spectrometer (Aminco-Bowman® Series 2 Spectrometer; Spectronic Instruments, Rochester, NY, USA). Excitation was provided at 590 nm rather than at 620 nm, the maximal absorbance of phycocyanin, the major pigment of the phycobilisome, to allow measurement of the complete fluorescence peak. (B) Sorting was done by FACSVantage™ SE (BD Biosciences) employing FL4 (633 nm excitation and emission with a band-pass filter at 660/20 nm). The arrows indicate the fluorescence gating for cell collection during the sorting procedure. (C) Cultures and (D) absorption spectra of whole cells representing wild-type (1) or nonbleaching mutants: the previously isolated nblRΩ (2) and the newly isolated mutants M56 (3) and M104 (4). Cultures were starved for 48 h. Fluorescence and absorbance spectra (A and D, respectively) were measured at equal cell density; however, for clarity, spectra were shifted along the y-axis. All data shown are taken from one experiment that is representative of three similar experiments. A, absorbance.

ing the excitation of chlorophyll via the Soret band, using a blue laser (e.g., 407 nm). Similar excitation in combination with the use of specific band-pass filters (420–440 nm and 460–490 nm) could be used to differentiate between chlorophyll *a* and chlorophyll *b*, respectively. Such conditions are suitable for isolating mutants of algal species containing both chlorophyll *a* and *b* or for the examination of protoplasts of higher plants. Finally, mutants of organisms in which carotenoids serve as the major light-harvesting pigments could also be isolated; for example, the level of fucoxanthine, the predominant carotenoid in certain algae, is reflected in a broad fluorescence band centered around 530 nm.

In summary, the four genes isolated thus far and further characterization of their products (11–15) provided some clues to the mechanism of degradation of the phycobilisome, the highly abundant macromolecular structure comprising the cyanobacterial light-harvesting complex. The isolation of novel nonbleaching mutants, however, seems to be a rate-limiting step in the study of the degradation process and its regulation. FACS-mediated selection of nonbleaching mutants will undoubtedly lead to a better understanding of the molecular mechanisms underlying the modulation of light harvesting in response to environmental cues by facilitating the isolation of novel mutants.

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Rapid analysis of oxysterols by HPLC and UV spectroscopy

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Oxidized derivatives of cholesterol are an important group of molecules with potent pharmacological properties (1) that have been implicated in atherosclerosis and other age-related diseases (2). The accurate identification and quantification of oxysterols is complicated by numerous factors, including low sensitivity of detection, interference by other compounds, tedious analytical methods, and instability during alkaline hydrolysis. Cholesterol and most oxysterols have ultraviolet (UV) absorption maximums below 200 nm and are extremely hydrophobic. This creates a unique chromatography problem because most solvents capable of solubilizing oxysterols (with dielectric constants below 30) absorb light strongly between 190 and 200 nm and their use reduces the sensitivity of detection by UV absorption. Water and acetonitrile have low UV absorption profiles above