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Photoheterotrophic Growth of *Agmenellum quadruplicatum* PR-6

DAVID H. LAMBERT AND S. EDWARD STEVENS, JR.*

Department of Molecular and Cell Biology, The Pennsylvania State University, University Park, Pennsylvania 16802

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The unicellular cyanobacterium *Agmenellum quadruplicatum* PR-6 grows in the presence of light on agar containing 10 μM 3-(3,4 dichlorophenyl)-1,1-dimethylurea and 1 to 30 mM glycerol. A derivative strain, PR-6G2, was tolerant of 100 mM glycerol. Photoheterotrophic growth conditions had little effect on transformation competence but did decrease the viability of single cells plated onto agar, particularly cells of the parent strain.

Photoheterotrophy, the light-dependent growth of a photosynthetic organism on a carbon source other than CO_2, is not only of intrinsic physiological interest but is a prerequisite for studies of mutants with impaired CO_2 fixation. Photoheterotrophic growth of nonmutants was demonstrated previously by providing a reduced carbon source while either eliminating CO_2, inhibiting photosystem II, or providing light too dim for normal photosynthesis (2, 4, 5, 9). Most photoheterotrophic cyanobacteria use glucose, but some may also use or require another carbon source (5). To allow growth, sufficient amounts of a particular carbon source must be taken up and metabolized to normal storage products without severely repressing necessary pathways. The unicellular cyanobacterium *Agmenellum quadruplicatum* PR-6 (8) has been reported to grow photoheterotrophically on glycerol (5) and, more slowly, on glucose (5, 9). This organism is presently the only photoheterotrophic cyanobacterium in which transforming DNA has been expressed in both the chromosome and in plasmids (1, 7; R. D. Porter, Crit. Rev. Microbiol., in press). The genes encoding both subunits of strain PR-6 ribulose biphosphate carboxylase-oxygenase are now available (D. H. Lambert and S. E. Stevens, Jr., unpublished data), and the genes encoding photosystem II subunits A, B, C, and D are currently being isolated (D. A. Bryant, personal communication). The purpose of this paper is to delineate photoheterotrophic growth in *Agmenellum* species so that this organism might be adapted for site-specific mutagenesis of these genes, which are essential for autotrophic growth.

The *A. quadruplicatum* strains used here were PR-6 (8) and its glycerol-tolerant derivatives, PR-6G1 and PR-6G2. The strains were grown on various modifications of medium A (6) to determine (i) the ability of strain PR-6 to grow on agar amended with 3-(3,4 dichlorophenyl)-1,1-dimethylurea (DCMU) and certain carbon sources, (ii) the mutation rates of strain PR-6 to the PR-6G1 and PR-6G2 phenotypes, (iii) the relative growth rates of strains PR-6 and PR-6G2 under autotrophic and heterotrophic conditions, (iv) the effects of the nitrogen source on photoheterotrophic growth, and (v) the effects of photoheterotrophy on transformation competence and expression.

Carbon sources. Strain PR-6 inoculum was spread on agar containing 0 or 10 μM DCMU and either glucose, fructose, sodium malate, or sodium citrate at both 10- and 50-mM concentrations. Growth on glycerol was likewise screened at concentrations of 3, 10, 30, and 100 mM. All media contained 1 g of sodium nitrate per liter.

Mutation rates. Appropriate dilutions of strain PR-6 inoculum were spread on agar containing either 0 or 100 mM glycerol. The glycerol-tolerant mutants generated were further screened on agar containing 10 mM glycerol–10 μM DCMU to differentiate between the phenotypes which could (PR-6G2) and could not (PR-6G1) grow on glycerol as a sole reduced carbon source.

Generation times. Liquid medium containing 4 mM urea was amended with 0, 1, 3, 10, or 30 mM glycerol and 0 to 10 mM DCMU. Four or five 20-ml replicate tubes were inoculated with strains PR-6 and PR-6G2 to an initial optical density at 550 nm of 0.02 (approximately 10^6 cells per ml) and incubated at 38°C. Optical density values were recorded every 6 h for cells growing autotrophically and every 6 or 12 h for cultures containing DCMU. After correcting for deviations in absorbance at higher optical densities, doubling times were calculated for exponential regions of the resulting growth curves. The optical density values for each replicate were log transformed and were regressed on their corresponding observation times to determine slopes for that replicate. The slope values were used for statistical analyses, and the average slope for each treatment was used to calculate doubling times.

Nitrogen sources. Liquid medium amended with 10 mM nitrogen as urea or sodium nitrate with or without 10 mM glycerol–10 μM DCMU was inoculated with strain PR-6G2, incubated at 40°C, and analyzed as above.

Transformation efficiency. Cultures of strains PR-6 and PR-6G2 were grown in liquid medium with and without 10 mM glycerol–10 μM DCMU to an optical density of 0.8. Then 9 volumes of these cells was mixed with 1 volume of DNA (2 μg of Tris-EDTA buffer [pH 8.0] per ml) from a Str^r PR-6 mutant (6). After 1 h of incubation, the mixtures were diluted 100-fold, and four replicates of 100 μl each were spread onto medium A plates with or without 10 mM glycerol–10 μM DCMU. In addition, nondiluted portions of the two PR-6 mixtures were spread onto glycerol-DCMU plates. Simultaneously, samples of these mixtures at higher dilutions were plated onto four replicates each of A medium without glycerol-DCMU to determine the number of CFUs. After individual colonies appeared, those plates not used to determine CFUs were underlaid with 2 ml of 0.4% agarose containing 50 μg of streptomycin per ml agar-agarose, and Str^r colonies were counted after the background Str^s colonies had lost their color.

On agar plates containing 10 μM DCMU, strain PR-6 did

* Corresponding author.

TABLE 1. Doubling time of *A. quadruplicatum* PR-6 and PR-6G2 grown in liquid medium on urea at 38°C with or without 10 μM DCMU plus glycerol

Glycerol concn (mM) in A medium	Mean doubling time (h) of indicated strain grown in:[a]			
	No DCMU		10 μM DCMU	
	PR-6	PR-6G2	PR-6	PR-6G2
0	4.8 A	4.7 A	NG[b]	NG
1	4.9 A	5.2 A	19.6 D	17.8 C
3	4.6 A	5.1 A	18.2 CD	13.3 B
10	4.7 A	5.0 A	17.8 C	12.6 B
30	4.6 A	4.8 A	17.8 C	12.2 B

[a] Means followed by the same capital letter do not differ at the 5% level of statistical significance by the Duncan modified significant difference test (3); means followed by C also do not differ significantly from means followed by CD.

[b] NG, No growth.

not grow perceptibly with glucose, fructose, sodium citrate, or sodium malate as the sole carbon source at either 10- or 50-mM concentrations. PR-6 did grow on heavily inoculated DCMU plates containing glycerol, but growth and pigmentation diminished progressively at concentrations of 10 mM and above. At 30 and 100 mM glycerol, a number of nearly normal, dark green colonies developed in the pale green background.

The apparent mutation rate of wild-type PR-6 cells to glycerol-tolerant cells was 8×10^{-6} per CFU. This is an apparent rate, because glycerol did not completely inhibit growth and some mutations to tolerance may have occurred in generations subsequent to plating. Of 227 mutant colonies subcultured, 24 colonies were able to grow on glycerol-DCMU agar. Thus, the maximum mutation rates to the PR-6G1 and PR-6G2 phenotypes were about 7 and 0.8 per 10^6 CFUs, respectively. When 10^8 cells of strain PR-6G1 were spread on 100 mM glycerol-DCMU plates, no dark green colonies developed, which indicated a mutation rate of less than 1 per 10^8 CFUs for the change from type G1 to type G2 and implied that these types are incompatible. In strain PR-6G1 it is likely that one or more steps in glycerol utilization were lost, preventing glycerol use and toxicity. In PR-6G2, glycerol toxicity was either reduced or the metabolism of glycerol was expedited so that toxic products accumulated to lower concentrations.

At 38°C in liquid medium without DCMU, the doubling times of strains PR-6 and PR-6G2 were both about 5 h and did not differ significantly over a range of glycerol concentrations (Table 1), which indicated that the type G2 mutation had not occurred in a critical pathway. In a preliminary trial at 30°C, slight but significant growth reductions occurred at 1 mM glycerol and increased at higher concentrations (data not shown). In media containing 10 μM DCMU, strain PR-6 doubled in a minimum time of 18 h, and strain PR-6G2 doubled in 12 h (Table 1). Growth of strain PR-6G2 on 1 and 3 mM glycerol ceased abruptly when about 1.4×10^7 cells per μM glycerol had been produced. Whole-cell spectral scans indicated that growth on glycerol-DCMU reduced pigment levels in cells, that phycocyanin was more affected than chlorophyll, that strain PR-6 was more affected than PR-6G2, and that pigmentation recovered partially when glycerol was exhausted (data not shown).

At 40°C, strain PR-6G2 responded differentially to N sources under autotrophic and photoheterotrophic conditions (Fig. 1). Without DCMU, there was rapid initial growth with both N sources (2.5-h doubling times), followed by a period of slower logarithmic increase during which growth was faster on nitrate than on urea. Presumably, urea at 10 mM N was toxic to the rapidly growing photoautotrophic cells, because the threshold for ammonium toxicity of strain PR-6 is circa 4 mM (S. E. Stevens, Jr., unpublished data). In contrast, cells with glycerol in the presence of DCMU grew more rapidly on urea over the course of the experiment than on nitrate.

When mixed with DNA from Str^r PR-6 cells, all of the cell treatments were competent regardless of strain or previous carbon source (Table 2). However, plating transformed cells onto glycerol-DCMU agar reduced total numbers of Str^r colonies. In a separate study, the viabilities of PR-6 and PR-6G2 cells from an inoculum grown on CO_2 were 11 and 28% of normal when plated on agar containing 1 mM glycerol and 0.1 μM DCMU. When the inoculum was grown on glycerol-DCMU, the corresponding relative viabilities were 4 and 6% (data not shown). This approximates the reduction in numbers of strain PR-6G2 transformants. When plated onto glycerol-DCMU, the number of PR-6 transformants was very low with the CO_2 pretreatment. No Str^s or Str^r colonies developed from strain PR-6 inoculum grown in and plated onto media with 10 mM glycerol and 10 μM DCMU.

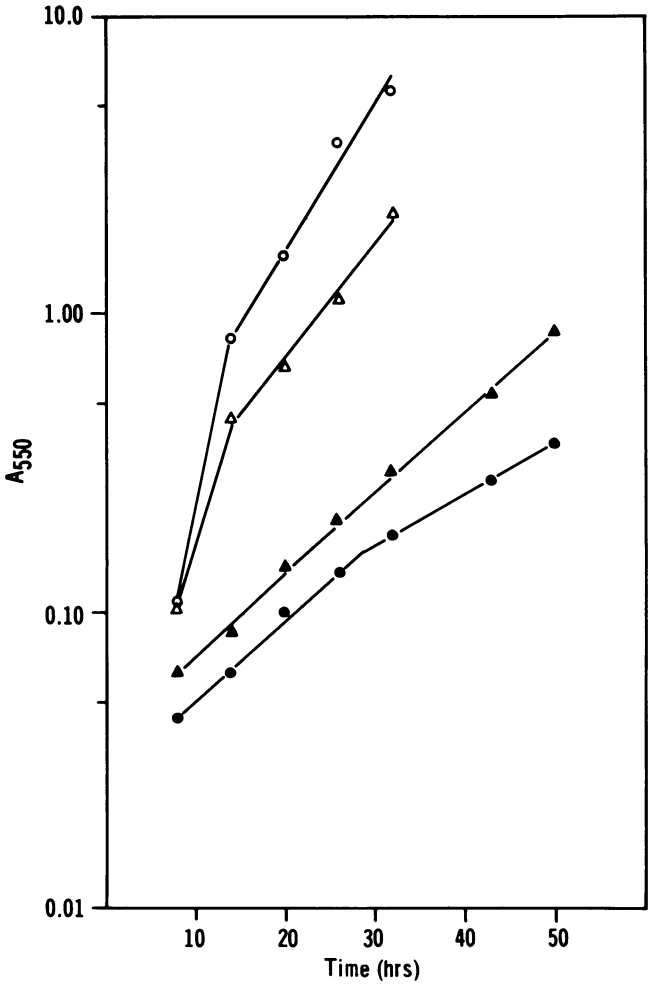

FIG. 1. Typical growth of *A. quadruplicatum* PR-6G2 in liquid media at 40°C with (●, ▲) or without (○, △) 10 mM glycerol–10 μM DCMU with 10 mM nitrogen as nitrate (○, ●) or urea (△, ▲).

TABLE 2. Transformation rates for strains PR-6 and PR-6G2 grown on CO_2 or 10 mM glycerol–10 μM DCMU before and after transformation with DNA from a streptomycin-resistant PR-6 mutant

Strain	Carbon source		No. of transformants per 10^3 CFU
	Pretransformation	Posttransformation	
PR-6	CO_2	CO_2	3.3
	CO_2	Glycerol	0.01
	Glycerol	CO_2	0.7
	Glycerol	Glycerol	NS[a]
PR-6G2	CO_2	CO_2	1.3
	CO_2	Glycerol	0.7
	Glycerol	CO_2	1.8
	Glycerol	Glycerol	0.1

[a] NS, None seen.

These results may be compared with those for the cyanobacterium *Synechocystis* strain 6803 (J. G. K. Williams and L. McIntosh, personal communication). In this species, glucose was more toxic to single cells on agar than to cells in liquid culture. In contrast, DCMU reduced but did not eliminate glucose toxicity. A similar mutant (6803-G) that was tolerant of glucose was recovered from a photoheterotrophic culture and apparently grew as well as the wild-type strain under photoautotrophic conditions. Although both strains were transformed to kanamycin resistance when grown photoautotrophically, only the tolerant mutant was transformed in photoheterotrophic culture. Thus, the potential for mutations to tolerance of reduced carbon sources may be a general phenomenon among photoheterotrophic cyanobacteria. Amelioration of toxicity, possibly caused by catabolic repression, appears to be an important and sometimes essential component of photoheterotrophy, and tolerant mutants perhaps should be sought when photoheterotrophic systems are developed.

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