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Genetics

Transformation in *Agmenellum quadruplicatum*

(genetic exchange/cyanobacteria/blue-green algae/nitrite reductase)

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ABSTRACT **A DNA-mediated transformation system for the blue-green alga *Agmenellum quadruplicatum*, strain PR-6, is described and characterized for DNA concentration dependence, dependence on time of exposure to DNA, phenotypic expression, sensitivity to various enzymes, and competence. The stability of the transformants has been investigated, and genetic backcross and selfing experiments have been performed. This system fulfills all of the criteria established for the well-characterized transformation systems in heterotrophic bacteria and demonstrates significant similarities to at least one of these systems for all characteristics examined. The efficiency of transformation is high. This system fills a need for a well-characterized genetic system in an oxygen-evolving photoautotroph. We have used it to transform a strain with a mutational lesion in assimilatory nitrogen metabolism to a wild-type genotype.**

The blue-green algae (cyanobacteria) provide an excellent model system for the study of photoautotrophic metabolism. They carry out oxygen-evolving photosynthesis and assimilate oxidized nitrogen in a fashion similar to that of higher plants and are amenable to many of the techniques utilized in the study of heterotrophic bacteria. A limiting factor in studying the metabolism of these organisms has been the lack of a system for genetic exchange. Although there have been reports of genetic exchange in blue-green algae (reviewed in ref. 1), only the transformation system in *Anacystis nidulans* has been reasonably well characterized (2, 3).

We have found that the blue-green alga *Agmenellum quadruplicatum*, strain PR-6, possesses an efficient, naturally occurring mechanism for the uptake and integration of exogenous DNA in a process like transformation in other bacteria (4). Here we characterize this system for sensitivity to DNase, dependence on concentration of DNA, dependence on time of exposure of cells to DNA, competence, and expression of newly incorporated genetic material. The PR-6 transformation system is similar to the standard heterotrophic bacterial transformation systems in all respects thus far tested.

We also describe an initial experiment wherein a physiologically well-characterized mutant of PR-6, called AQ-6 (5–8), was transformed with parental DNA. AQ-6 reduces nitrate to nitrite but its reduction of nitrite is impaired, resulting in the accumulation of nitrite in the medium. The mutant grows normally in the presence of ammonia. After transformation at least two colonial phenotypes were recovered. Genetic dissection of inorganic nitrogen assimilation in blue-green algae is now feasible.

MATERIALS AND METHODS

Strains, Media, and Growth. The unicellular blue-green alga *Agmenellum quadruplicatum*, strain PR-6 (American Type Culture Collection, 27264; Pasteur Culture Collection, 7002) was isolated by Van Baalen (9). The *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced mutant of PR-6 called AQ-6 was isolated by Stevens and Van Baalen (5). Liquid cultures of PR-6 were grown in medium A (10) and those of AQ-6 were grown in medium A plus 150 μg of NH_4Cl per ml and lacking $NaNO_3$ (8) in a water bath maintained at 39°C. Continuous agitation and CO_2 were provided by bubbling 1% CO_2/99% air through each culture. Illumination was provided by four F24T12 CW/HO fluorescent lamps providing a total of 580 μeinsteins cm^{-2} sec^{-1} incident on each culture.

Agar cultures of PR-6 or AQ-6 were grown with the appropriate nitrogen source on medium A solidified with 1.5% agar (Difco, 0140) at 32 ± 2°C under four F96T12 CW fluorescent lamps providing 250 μeinsteins cm^{-2} sec^{-1} of light intensity incident on each plate. The edges of each plate were wrapped with Parafilm to protect the contents from desiccation.

Growth and cell concentration of PR-6 and AQ-6 were routinely measured turbidimetrically with a Spectronic 20 colorimeter at 550 nm. An average concentration of 4.2×10^7 cells per ml was indicated by an optical density of 0.82 in a 22-mm culture tube.

DNA Preparation. DNA from PR-6 and from a streptomycin-resistant derivative (see below) were purified by the procedure of Marmur (11).

Standard Transformation Procedure. Nine volumes of recipient cells grown to approximately 4×10^7 cells per ml were mixed with 1 vol of DNA in 0.15 M NaCl/0.015 M Na_3citrate and incubated for the desired length of time before addition of 1 vol of DNase I to a final concentration of 10 μg/ml. The cells were then plated on the surface of medium A agar plates and incubated. After time for expression of streptomycin resistance (see Fig. 1), the plates were sprayed with a sterile 2% solution of streptomycin sulfate to give a final concentration of approximately 50 μg/ml, and the incubation was continued. Streptomycin-resistant (Str^r) transformants appeared 3–4 days after the addition of the drug.

Reagents. The streptomycin sulfate was obtained from Eli Lilly and the other drugs were obtained from Sigma. DNase I was from Sigma, RNase was from Worthington, and proteinase K was from Merck. All other chemicals were reagent grade.

Mutant Isolation. Spontaneous mutants of PR-6 resistant to streptomycin were isolated by plating large numbers of cells on plates containing a concentration gradient of the drug. Mutation frequencies varied between 1 and 3×10^{-9} for the streptomycin resistance. Although not used in this study, we have isolated spontaneous mutants resistant to erythromycin, rifamycin, spectinomycin, and nalidixic acid at similar frequencies. The Str^r mutants used in this study were routinely tested on agar plates containing 100 μg of streptomycin sulfate per ml and they remained stably Str^r after extensive subculturing on nonselective media.

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Abbreviations: Str^r, streptomycin-resistant; Str^s, streptomycin-sensitive.

RESULTS

Purified DNA. The purified DNA preparations had the spectral characteristics previously published for PR-6 DNA (12) and were typical of DNA purified from various sources (11). Analysis on agarose gels indicated a reasonably homogeneous molecular weight of 12–15 × 10^6.

Sensitivity to Enzymes. The criterion that a genetic exchange is due to DNA-mediated transformation is that it is prevented by DNase (13). We tested the apparent transformation of PR-6 for sensitivity to DNase, RNase, and proteinase K. It was completely sensitive to the action of DNase but was unaffected by RNase or proteinase K (Table 1).

Stability of Transformants and Backcross. The Str^r transformants obtained were stably Str^r after extensive subculture on nonselective media. Furthermore, DNA purified from a Str^r transformant transformed the streptomycin-sensitive parental strain with the same efficiency as did DNA from the original Str^r mutant (data not shown).

$CaCl_2$-Mediated Transformation. Some prokaryotes that lack a natural mechanism for the uptake of exogenous DNA can be transformed by treating them with a $CaCl_2$ regimen (4). A culture of exponentially growing PR-6 cells (4 × 10^7 cells per ml) was carried through the $CaCl_2$-mediated transformation procedure designed for *Escherichia coli* by Cosloy and Oishi (14). PR-6 Str^r DNA was used at 0.14 μg/ml. The $CaCl_2$-treated cultures were incubated with DNase immediately upon completion of the $CaCl_2$ procedure. The cells were then diluted back to their original density and plated. The $CaCl_2$ transformation procedure yielded 2.5 × 10^3 Str^r transformants per ml and there were 1.1 × 10^7 viable cells per ml after the $CaCl_2$ treatment. A standard transformation experiment was run concurrently with the same batch of cells and yielded 7 × 10^4 Str^r transformants per ml from 4 × 10^7 viable cells per ml. Thus, the naturally occurring transformation system yielded 10-fold more transformants per viable cell than did the $CaCl_2$ procedure. Under the circumstances, it is difficult to say whether the transformants seen from the $CaCl_2$ procedure resulted from $CaCl_2$-mediated uptake of DNA or from DNA uptake by the more efficient physiological mechanism.

Phenotypic Expression of Streptomycin Resistance. Cells that have been transformed to drug resistance usually require a period of time for the phenotypic expression of that drug resistance before they are no longer sensitive to a challenge by the drug. The phenotypic expression of streptomycin resistance has been documented for the case of transformation in pneumococcus (*Streptococcus pneumoniae*) (15).

Table 1. Sensitivity of transformation to enzymes

Sample	DNA	Enzyme added: At 0 time	Enzyme added: At 30 min	Str^r colonies/ml
1	−	None	DNase	24/0.5 ml*
2	+	None	DNase	1.2 × 10^5
3	+	DNase	None	7/0.5 ml*
4	+	RNase	DNase	1.4 × 10^5
5	+	Proteinase K	DNase	1.3 × 10^5

One volume of PR-6 Str^r DNA at 34.5 μg/ml in 0.15 M NaCl/0.015 M Na_3citrate was mixed with 1 vol of each enzyme at 100 μg/ml and immediately thereafter with 9 vol of PR-6 at 4 × 10^7 cells per ml. After 30-min incubation at 39°C, DNase was added to the tubes as indicated. Dilutions were plated and incubated 40 hr at 32 ± 2°C before challenge with streptomycin and continued incubation. The background levels of Str^r colonies (samples 1 and 3) represent spontaneous mutation frequencies of 2.5 × 10^{-9} and 1 × 10^{-9}, respectively, when the number of cells present at the time of streptomycin challenge is considered.

* These numbers represent colonies counted per volume plated; the other numbers have been calculated from dilution platings.

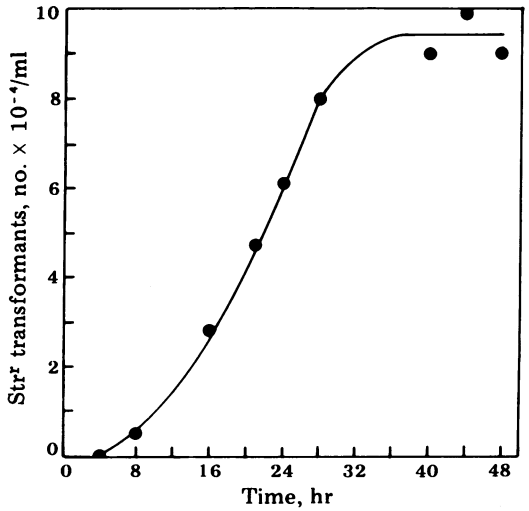

FIG. 1. Expression of streptomycin resistance in transformants. One volume of PR-6 Str^r DNA at 0.14 μg/ml in 0.15 M NaCl/0.015 M Na_3 citrate was mixed with 9 vol of PR-6 at 4 × 10^7 cells per ml. After incubation for 3 hr at 39°C, 1 vol of DNase was added to a final concentration of 10 μg/ml. Platings were incubated at 32 ± 2°C, and selected dilutions were challenged with streptomycin at the indicated times before continuing the incubation at 32 ± 2°C.

Phenotypic expression of streptomycin resistance in PR-6 transformation is shown in Fig. 1. Significant numbers of transformants were observed after 8 hr of incubation at 32 ± 2°C prior to the streptomycin challenge. The increase in transformants was then approximately linear with the time of expression allowed until about 28 hr; a plateau was reached prior to 40 hr. The incubation for 40 hr at 32 ± 2°C represents about eight cell doublings under these conditions. Subsequent experiments involved 40–48 hr of expression before challenge with streptomycin.

Dependence on DNA Concentration. We tested the DNA concentration of PR-6 transformation to streptomycin resistance over a range from 0.002 to 13.8 μg/ml. At DNA concentrations from 0.002 to 0.05 μg/ml, the number of transformants obtained was a linear function of the DNA concentration (Fig. 2).

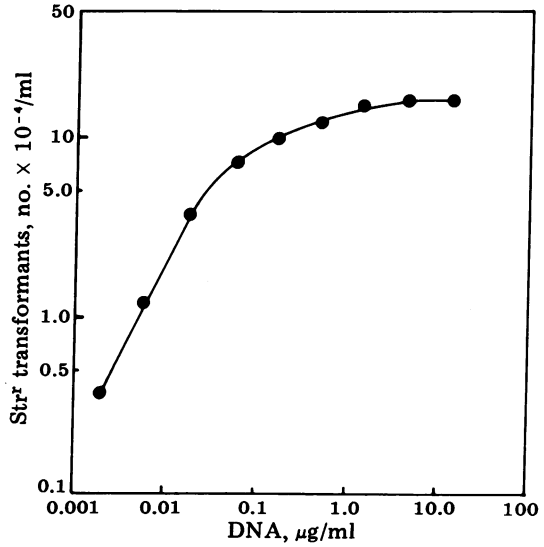

FIG. 2. DNA concentration dependence of transformation. A series of 1:3 dilutions of PR-6 Str^r DNA in 0.15 M NaCl/0.015 M Na_3 citrate was made. Each DNA dilution was used in a standard transformation experiment with the same culture of competent PR-6 and 3 hr of exposure of cells to DNA before addition of DNase.

Above 0.06 μg/ml the response began to saturate, reaching a plateau at approximately 1 μg/ml of DNA in the transformation mix. Half-saturation of the transformation reaction occurred at about 0.08 μg/ml. The DNA concentration response curve for PR-6 transformation is similar to those described for certain heterotrophic bacteria capable of transformation (16–18).

Dependence on Time. Two experiments to test the dependence of PR-6 transformation frequency on the time of exposure of recipient cells to DNA are shown in Fig. 3. The level of transformants increased quickly and reached a plateau by 30 min of exposure of recipient cells to DNA. There was a short lag, 1–2 min, before DNase resistant transformants appeared in the population. This lag was followed by a period of 3–4 min during which transformation was a linear function of DNA exposure time.

Because this linear response region was fairly short, we thought it might reflect the emergence of some impediment to binding or uptake shortly after the start of exposure. Therefore, in a separate experiment we added a second dose of DNA to the cells after 10 min of exposure to the first DNA dose. Cell samples with and without the second DNA addition were treated with DNase after 20 min of total exposure time. The result (data not shown) was that cells receiving the second dose of DNA had twice as many transformants as cells that received only one dose of DNA. This cannot be explained as a function of DNA concentration dependence because this experiment was done at a DNA concentration such that a doubling of concentration should result in no more than a 25% increase in transformants. The increment in transformants with a second dose of DNA also indicates that the cells are not impaired in their ability to bind or takeup DNA. It seems most likely that the DNA is being degraded or altered in such a way that it is unavailable for transformation.

Competence. Initial transformation experiments were performed with mid-exponential phase recipient cells, largely as a matter of convenience. We later examined the growth phase dependence of the ability of cells to become competent for transformation by periodically removing a sample of cells from a growing culture and exposing it to DNA for a short time period. The two experiments shown in Fig. 4 indicate that the ratio of transformants to viable cells is essentially independent of cell number throughout exponential growth. With exponentially growing cells there is no pronounced dependence of competence on cell number. Cells in stationary phase showed decreased but still substantial levels of competence.

Dependence of Transformation on Light. Our normal transformation protocol involved incubation of a mixture of DNA and recipient cells at the normal growth temperature with high-intensity illumination and aeration with CO_2 in air. We also tested PR-6 transformation for its direct dependence on light for DNA binding and uptake. A culture of competent cells was divided and part of it was incubated for 15 min in a foil-wrapped tube at 39°C with normal CO_2 bubbling. Samples of cells grown in light and in dark were then exposed to DNA in culture tubes with and without foil wrapping. These four transformation cultures were then treated with DNase and incubated an additional 15 min under the transformation conditions before plating. The cells were challenged with streptomycin after 40 hr of incubation at 32 ± 2°C for expression. We obtained about 2.5×10^4 Str^r transformants per ml with all four possible combinations of light and dark incu-

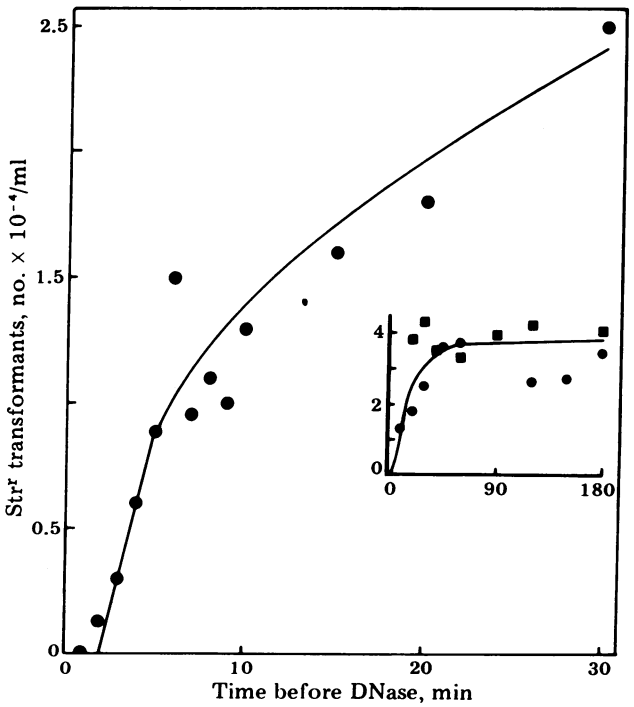

FIG. 3. Time dependence of transformation. One volume of PR-6 Str^r DNA and 9 vol of PR-6 at 4×10^7 cells per ml were mixed to give a final DNA concentration of 0.14 μg/ml. This mixture was incubated at 39°C. At the indicated times, an aliquot of the transformation mixture was added to an equal volume of DNase in A medium to give a final DNase concentration of 10 μg/ml. After 10 min of continued incubation at 39°C with light and CO_2, platings were made and incubated 40 hr at 32 ± 2°C before challenge with streptomycin. (*Inset*) Same experiment plus a second experiment (different symbols) run for an extended time period.

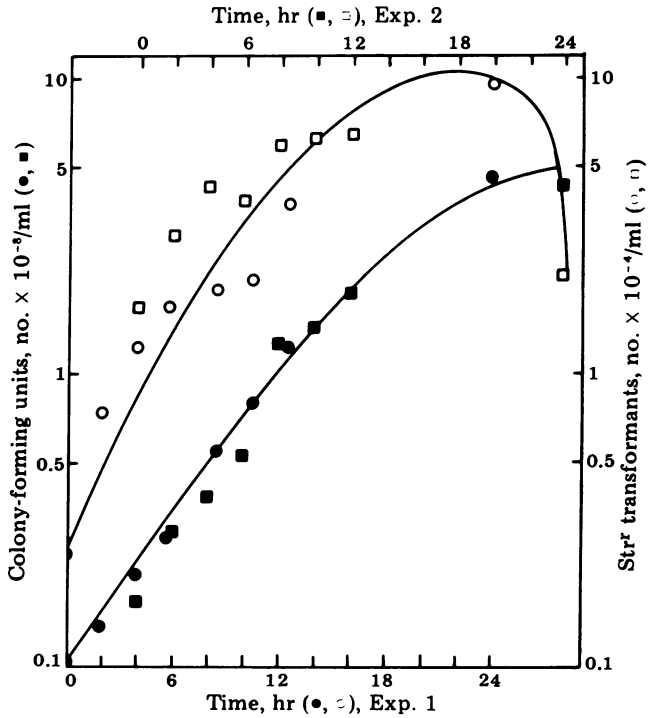

FIG. 4. Growth phase dependence of competence. A culture of PR-6 at 2×10^7 cells per ml was maintained at 39°C with fluorescent lighting and bubbling with 1% CO_2/99% air. At the indicated times, 0.9 ml of cells was added to 0.1 ml of PR-6 Str^r DNA to give a final DNA concentration of 0.14 μg/ml. After 10 min of exposure of cells to DNA, 0.1 ml of DNase was added to give a final DNase concentration of 10 μg/ml. These cultures were incubated 15 min more before plating. The cells were challenged with streptomycin after 40 hr of incubation at 32 ± 2°C.

bation prior to and during transformation. Continuous exposure to light therefore is not necessary for DNA binding and uptake by PR-6.

Frequency of Transformation. Utilizing nearly saturating concentrations of donor DNA, we routinely obtained between 2×10^4 and 1.5×10^5 Str^r transformants per ml from a recipient culture containing 4×10^7 viable cells per ml. This is a frequency of 5×10^{-4} to 3.7×10^{-3} per recipient cell for a single genetic marker. This frequency should be readily usable for genetic analysis in this organism.

AQ-6 Transformation. AQ-6 is a mutant of PR-6 (5–8) that is deficient in nitrite reductase. When AQ-6 is grown on a medium containing ammonia, it is essentially indistinguishable from PR-6 and forms large forest-green colonies on agar plates. When it is grown on a medium containing nitrate as the sole nitrogen source, it produces small yellow-green colonies that are easily distinguishable from PR-6.

We have transformed the lesion in AQ-6 nitrogen metabolism back to wild type. PR-6 Str^r DNA was added to AQ-6 cells. Platings were done on medium A with ammonia and later challenged with streptomycin to measure the level of Str^r transformants. Other platings were done on medium A with nitrate. After several days of incubation, the large forest-green transformants were easily distinguishable from the backgrounds of small yellow-green AQ-6 parental colonies. We obtained 1.0×10^4 Str^r transformants per ml and 7.7×10^3 nitrite-reductase positive transformants per ml, starting with a culture of AQ-6 that contained 2.4×10^7 viable cells per ml.

Selfing Experiment. One report of transformation in the blue-green alga *Anacystis nidulans* claimed that transformation itself results in the production of mutational lesions (19). In contrast, a subsequent report about the same organism indicated that Str^r recipient cells did not arise when streptomycin-sensitive (Str^s) donor DNA was utilized (3). To examine this possibility for Str^r transformation in PR-6, we performed a selfing experiment with PR-6 that included AQ-6 transformation to ensure the biological activity of the PR-6 Str^s DNA. We have not done a selfing experiment in AQ-6 *per se*. Competent cultures of both PR-6 and AQ-6 were transformed with DNA from the Str^s parental PR-6 as well as DNA from a Str^r derivative of PR-6. The PR-6 Str^s DNA yielded nitrite reductase-positive transformants of AQ-6 at high levels but failed to produce Str^r colonies with either AQ-6 or PR-6 (Table 2). Thus, transformants seen with PR-6 transformation were the result of the incorporation of a streptomycin-resistance allele into the recipient genome and not the result of mutation production by the transformation process.

Table 2. Selfing experiment

Recipient strain	DNA	Str^r, cells/ml	Nitrite reducing, cells/ml
PR-6	None	0/0.2 ml*	—
	PR-6 Str^s	1/0.2 ml*	—
	PR-6 Str^r	7.0×10^4	—
AQ-6	None	1/0.2 ml*	0/0.2 ml*
	PR-6 Str^s	3/0.2 ml*	2.6×10^4
	PR-6 Str^r	5.2×10^3	1.9×10^4

Nine volumes of exponentially growing AQ-6 or PR-6 at 4×10^7 cells per ml was added to 1 vol of PR-6 Str^s or Str^r DNA to give final DNA concentrations of 0.4 and 0.14 μg/ml, respectively. These cultures were incubated at 39°C for 3 hr. One volume of DNase solution was added to give a final DNase concentration of 10 μg/ml. Platings were done on A medium with nitrate for PR-6 and on A medium with nitrate and A medium with ammonia for AQ-6. The platings of AQ-6 on A medium with ammonia and PR-6 on A medium with nitrate were challenged with streptomycin after 40 hr of incubation at 32 ± 2°C. The platings of AQ-6 on A medium with nitrate were not challenged with the drug. Str^r transformants were those surviving streptomycin challenge; while nitrite reducing transformants were scored as large forest green colonies when grown on A medium with nitrate.

* These numbers represent colonies counted per volume plated; the other numbers have been calculated from dilution platings.

DISCUSSION

We have described a DNA-mediated transformation system for *A. quadruplicatum*, strain PR-6, that is similar to such systems in several heterotrophic bacteria. The frequencies of transformation are high enough (5×10^{-4} to 3.7×10^{-3} per recipient cell for a single genetic marker) to make the system useful for genetic analysis in PR-6.

The PR-6 transformation system fulfills three of the basic criteria for transformation first set forth in the classic study by Avery *et al.* (13) on transformation with pneumococcus (*Streptococcus pneumoniae*). PR-6 transformation by purified DNA is totally sensitive to DNase and insensitive to RNase and proteinase K (Table 1). The transformants obtained with PR-6 are stable for the transformed genetic characteristic, and DNA from a PR-6 transformant gave further transformants in a backcross with the original recipient parental strain.

In recent years the number of transformable procaryotes has been extended by the use of $CaCl_2$ treatment of cells to force the uptake of DNA (4). Our attempts to use $CaCl_2$-mediated DNA uptake with PR-6 always gave fewer transformants than were obtained with untreated cells by the natural DNA uptake mechanism. Whether or not $CaCl_2$-mediated DNA uptake functions in PR-6 therefore is not clear.

We have not yet investigated the molecular nature of our drug-resistance mutants in PR-6 but for practical considerations we examined the phenotypic expression of streptomycin resistance in transformation. Streptomycin resistance becomes fully expressed phenotypically only after approximately eight normal cell doublings have occurred subsequent to the uptake of the donor DNA coding for streptomycin resistance (Fig. 1). Premature challenge of potential transformants with the drug results in a lower observed transformation frequency.

The dependence of PR-6 transformation on DNA concentration (Fig. 2) is similar to that described in pneumococcus (16), *Bacillus subtilis* (17), and *Hemophilus influenzae* (18). The half-saturation of DNA binding at a DNA concentration of about 0.1 μg/ml in these organisms has been interpreted as an indication of efficient DNA binding (20). It therefore would seem that PR-6 is also capable of binding DNA efficiently. The PR-6 transformation system differs significantly from that of *A. nidulans*, however, in that the latter system requires 20–40 μg of DNA per ml for maximal transformation (2, 3).

The dependence of transformation on the time of exposure of cells to DNA for PR-6 (Fig. 3) was similar to results obtained in pneumococcus (21) and *B. subtilis* (22). There was a short lag before the appearance of transformants followed by a linear response and a plateau. Both pneumococcus and *B. subtilis* show a short lag period followed by a linear response region. *H. influenzae*, however, responds quite differently, with the plateau region being reached in less than 2 min of cell exposure to DNA (18). The short duration of the response of PR-6 to DNA was not due to loss of competence because addition of a second increment of DNA gave further transformants. However, similar kinetics have been shown, in *B. subtilis*, to be due to the fact that the DNA was being inactivated by some action of noncompetent cells in the population (23). A similar mechanism could be operating in PR-6 transformation.

Our experiments concerning the growth phase dependence of competence for DNA binding and uptake indicate that exponentially growing cells routinely maintain some degree of

competence and that this competence is somewhat reduced when the cells enter stationary phase (Fig. 4). Other than the lack of a distinct peak of competence, this result is similar to that obtained with pneumococcus in which competence is a property of exponentially growing cells (24). It is in contrast to the results obtained with *B. subtilis* (17) and *H. influenzae* (25) in which competence is a property of stationary phase cells.

We investigated the possibility that DNA uptake in a photoautotroph might be directly dependent on light energy rather than on chemical energy accumulated in the cell. DNA uptake in PR-6 was not directly dependent on light.

One study of transformation in the blue-green alga *A. nidulans* found that mutations arose at a high frequency as a result of the recombination process (19); a second study indicated that this was not the case (3). To see if this was occurring in PR-6 transformation we ran a genetic selfing experiment (Table 2). We found that DNA purified from a Str^s strain of PR-6 failed to yield observable transformants when used as the donor DNA with a Str^s recipient strain. It is important to note that the cells used as recipients were shown to be competent by simultaneous transformation with DNA from a Str^r strain of PR-6. It was also shown that the DNA from the Str^s strain had biological activity by simultaneously transforming strain AQ-6 to a normal genotype for nitrite reductase (see below). We therefore conclude that our Str^r transformants are resulting from the incorporation of specific donor nucleotide sequences and not from mutagenesis promoted by recombination.

As described above, *A. quadruplicatum*, strain AQ-6, is a derivative of PR-6 that is deficient in nitrite reductase. One result of this deficiency is the formation of small yellow-green colonies by AQ-6 when grown on a medium containing nitrate as the only nitrogen source. Because these small yellow-green colonies are easily distinguishable from the large forest-green colonies formed by PR-6 under the same conditions, we have utilized this phenotypic manifestation to exploit the deficiency in nitrite reductase as a genetic marker in transformation. When AQ-6 was transformed with DNA from a Str^r derivative of PR-6, we obtained transformants restored to the large forest-green colony appearance of PR-6 at a frequency similar to that observed for transformants to streptomycin resistance. There have been some indications that the mutational lesion in AQ-6 is either pleiotrophic or the result of multiple lesions in that nitrate uptake is reduced as well as the activity of nitrite reductase (unpublished data). We have also noted at least one additional colony color phenotype in these transformation experiments, which may indicate a sorting out of more than one mutational lesion in AQ-6. It now appears that this transformation system will be of immediate usefulness in sorting out the genetics of assimilatory nitrogen metabolism. In the long term, it should also prove useful in studies designed to explore the genetics of other features of metabolism in an oxygen-evolving photoautotrophic organism.

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