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Chromosomal Transformation in the Cyanobacterium *Agmenellum quadruplicatum*

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Chromosomal transformation of *Agmenellum quadruplicatum* PR-6 (=*Synechococcus* sp. strain 7002) was characterized for phenotypic expression, for exposure time to DNA, and for dependence on DNA concentration with regard to Rif^r donor DNA. Exponentially growing cells of PR-6 were competent for chromosomal transformation. Competence decreased in cells in the stationary phase of growth or in cells deprived of a nitrogen source. Dark incubation of cells before exposure to donor DNA also decreased competence. Homologous Rif^r and Str^r DNA and heterologous *Escherichia coli* W3110 DNA were used in DNA-DNA competition studies, which clearly showed that DNA binding by PR-6 was nonspecific. DNA binding and uptake by PR-6 exhibited single-hit kinetics. Single-stranded DNA failed to transform competent cells of PR-6, and DNA eclipse was not observed, suggesting that double-stranded DNA was the substrate for the binding and uptake reactions during the transformation of PR-6. A significant improvement in transformation frequency was achieved by increasing the nitrate content of the culture medium and by lowering the temperature at which cells were exposed to donor DNA from 39°C (the optimal temperature for growth) to 30°C.

Stevens and Porter (29) demonstrated that the cyanobacterium *Agmenellum quadruplicatum* PR-6 possessed an efficient, naturally occurring mechanism for the uptake and integration of exogenous DNA. They characterized chromosomal transformation in PR-6 in terms of DNA concentration dependence, dependence on time of exposure to DNA, phenotypic expression, sensitivity to various enzymes, and competence. They also reported on the stability of transformants and performed genetic backcross and selfing experiments. Buzby et al. (4) followed up this work with the demonstration and initial characterization of plasmid transformation in PR-6. This work provided the basic knowledge that allowed the exploitation of plasmid transformation in PR-6 for subsequent work (5, 8, 23). Both chromosomal and plasmid transformation have been described in several species of unicellular cyanobacteria classified as *Synechococcus* sp. or as *Synechocystis* sp. (12, 22). Heterospecific transformation among the cyanobacterial genera *Synechococcus* and *Synechocystis* has also been demonstrated (30).

The ability to anticipate the genetic makeup of the product of transformation is the mark of its usefulness as a genetic tool. Knowledge of how the transforming DNA is processed and stabilized in the recipient is fundamental to this ability. The question of whether donor DNA is converted to the single-stranded state is central to an understanding of the mechanism of recombination resulting from chromosomal transformation. However, little is known about this process in cyanobacteria for either chromosomal or plasmid transformation. Herein, we further characterize chromosomal transformation in PR-6 by describing DNA-DNA competition and DNA eclipse experiments. It was essential for the performance of these experiments that we further optimize conditions for chromosomal transformation. It was also essential for the performance and interpretation of these experiments that we characterize a drug resistance marker in addition to the previously described Str^r marker (29). Thus, we report more optimal physiological conditions for chromosomal transformation and characterize the chromosomal transformation of Rif^r in terms of expression, DNA exposure time, and DNA concentration dependence.

MATERIALS AND METHODS

Strains, media, and growth. *A. quadruplicatum* PR-6 (ATCC 27264; PCC 7002) is a unicellular marine cyanobacterium isolated into axenic culture by Van Baalen (34). The Str^r mutant of PR-6 was previously isolated and described by Stevens and Porter (29). A spontaneous rifamicin-resistant (Rif^r) mutant of PR-6 was isolated by the same procedure used for the isolation of the Str^r mutant mentioned above. It was routinely tested on agar plates containing 3 μg of rifamicin per ml, and it remained stably Rif^r after extensive subculturing on nonselective medium. Liquid cultures of PR-6 and its drug-resistant mutants were grown in medium A, a minimal medium (28), in culture tubes (22 by 175 mm) at 30 to 39°C, as appropriate, in glass-sided water baths that were illuminated by four F72T12/CW fluorescent lamps. Agitation and CO_2 were provided by bubbling filtered 1% CO_2 in air via sterile glass tubes passing through sterile cotton plugs to the bottom of each culture tube. Agar plates were made with medium A supplemented with 5 rather than 1 g of $NaNO_3$ per liter and solidified with 1.5% agar (Difco Bacto-agar 0140). Plates were incubated at 32 ± 2°C under four F96T12/CW fluorescent lamps providing incident intensity of 250 μE cm^{-2} s^{-1}. The edges of each plate were sealed with Parafilm to prevent desiccation. *Escherichia coli* W3110, a λ^- F^- derivative of *E. coli* K-12, was grown in LB broth at 37°C with shaking (19). Growth and cell concentrations in liquid cultures were monitored turbidimetrically with a Baush & Lomb Spectronic 20 at 550 nm for PR-6 and its mutant progeny and at 650 nm for *E. coli*.

DNA isolation. DNA from a Str^r mutant of PR-6 and from *E. coli* W3110 was purified as described previously (29).

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During the progress of this work, a DNA purification procedure using the Rif^r mutant of PR-6 was developed that gave better yields of DNA. Exponentially growing cells (4 liters) were pelleted in 1-liter centrifuge bottles at 7,000 × *g* for 30 min in a Sorvall RC-3B centrifuge at room temperature. Pellets were suspended in a combined volume of 200 ml of lysis solution (10% sucrose, 50 mM Tris hydrochloride [pH 8], 10 mM disodium EDTA) and frozen. The solution was thawed at 37°C, lysozyme (Sigma Chemical Co.) was added to a final concentration of 10 mg/ml, and the mixture was incubated at 37°C for 30 min. Sarkosyl (2 g) was dissolved in 10 ml of distilled H_2O and added to the above-described solution, which cleared during a further 30-min incubation at 37°C. The solution was extracted once with a mixture of chloroform and isoamyl alcohol, the upper aqueous layer was removed, and $CsCl_2$ (1 g/ml; Kawecke Berylco Industries, Inc.) was added. The resulting solution was then centrifuged at 44,000 rpm in a 60 Ti rotor at 15°C for 40 h in a Beckman model L8-80M ultracentrifuge. Fractions were collected by bottom puncture, and all noticeably viscous material was pooled, made to 0.3 mg/ml with ethidium bromide, and centrifuged at 44,000 rpm in a Beckman 70.1 Ti rotor at 15°C for 40 h. Under UV illumination, two bands were visible. The upper chromosomal band was collected by side puncture, the ethidium bromide was *n*-butanol extracted, and the DNA was dialyzed extensively against 10 mM Tris chloride–1 mM EDTA (pH 7) buffer.

Transformation procedures. The standard transformation procedure and challenge with streptomycin sulfate (Eli Lilly & Co.) previously described by Stevens and Porter (29) were used unless otherwise indicated. For challenge with rifamicin (Sigma) after the standard transformation procedure, plates were sprayed with a sterile solution (300 μg/ml) to give a final concentration of approximately 3 μg/ml in the plate. A modified transformation protocol, described as follows, was used where necessary. Cells were grown to 10^8 cells per ml in medium A fortified with 5 g of $NaNO_3$ per liter. One volume of DNA was added to nine volumes of recipient cells at 27 to 30°C without bubbling. Varied times of incubation were allowed before addition of 1 volume of DNase I to a final concentration of 10 μg/ml. The cells were plated in 2.5 ml of 0.6% medium A overlay agar that had been tempered to 45°C. Plates were incubated without Parafilm. For challenge with streptomycin and rifamicin, 2.0 ml of 0.6% medium A agar containing 6 mg or 90 μg, respectively, of filter-sterilized drug was underlaid, per plate, with a sterile Pasteur pipette. Challenge with the appropriate drug was performed by underlay to avoid suspension and redistribution of resistant clones from surface colonies and to provide more even distribution of drug. Transformants appeared in 3 to 4 days after the addition of drug.

Crude lysis protocol for DNA eclipse experiments. Approximately 3 ml, containing about 10^8 cells per ml, was centrifuged at 8,000 × *g* for 10 min at 0°C. The pellet was suspended in 5 ml of ice-cold SSC (0.15 M NaCl, 0.015 M sodium citrate [pH 8.2]), centrifuged again, and suspended as before. After a subsequent centrifugation at the same velocity and temperature, the pelleted cells were suspended in 0.1 ml of 0.2× SSC (0.03 M NaCl, 0.003 M sodium citrate [pH 8.2]) at 0°C. The sample was then placed in a 60°C water bath, and 50 μl of a preheated (60°C) solution of 0.6 mg of lysozyme per ml in 10 mM Tris at pH 8.2 was added. After 2 min of incubation with occasional gentle stirring, 50 μl of preheated (60°C) sodium dodecyl sulfate (0.2%) in 3.1× SSC (0.47 M NaCl, 0.047 M sodium citrate [pH 8.2]) was added. After an additional 3 min of incubation, 1.8 ml of ice-cold SSC was added, and the preparation was promptly frozen at −20°C.

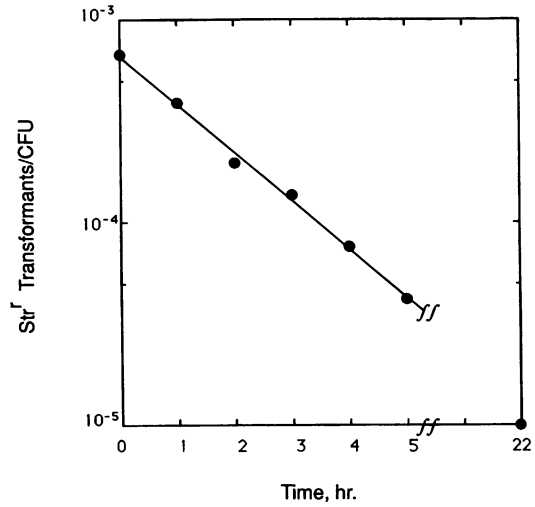

FIG. 1. Dependence of transformation on light. An exponentially growing culture of PR-6 was shifted to the dark but otherwise incubated under the usual conditions. Samples of cells (0.9 ml) were removed immediately and plated for the determination of CFU and transformed with Str^r DNA at a final concentration of 10 μg/ml, using the standard transformation procedure. This process was repeated at each of the indicated time points.

RESULTS

Physiological effects on transformation frequency in PR-6. Soluble competence factors that increase chromosomal transformation frequency have been demonstrated in the pneumococci (20, 32, 33). To evaluate the possible presence of a soluble protein competence factor(s) during transformation of PR-6, recipient cells were incubated for 24 h in medium A containing Pronase E (Sigma) at a final concentration of 10 μg/ml and transformed with Str^r and Rif^r DNA at various times. No statistically significant difference in transformation frequency between untreated cells of PR-6 and those treated with Pronase E was observed (data not shown).

Stevens and Porter (29) showed that competence for DNA transformation was continuous during exponential growth, with a small drop in transformation frequency observed upon entry of recipient cells into the stationary phase of growth. This observation was more carefully studied by use of nitrate deficiency (27) to induce cells to enter the stationary phase of growth while all other nutrients were sufficient for continued exponential growth. Nitrogen deficiency resulted in a decline in transformation frequency from 10^5 to 10^4 transformants per ml in the first 6 h after the beginning of nitrogen starvation, which paralleled the results previously reported (29).

Stevens and Porter (29) showed that DNA binding and uptake by PR-6 were not dependent on continued exposure to light. However, the effect of incubating cells in the dark before exposure to donor DNA was not examined. Transformability was tested over a 22-h period after the cells had been placed in the dark. An immediate and rapid decline in transformation frequency with time of dark preincubation occurred (Fig. 1). Roughly a 2-log decline in transformation frequency occurred, although CFUs decreased only by half. At the initial sampling point, there were 7.2 × 10^7 cells per

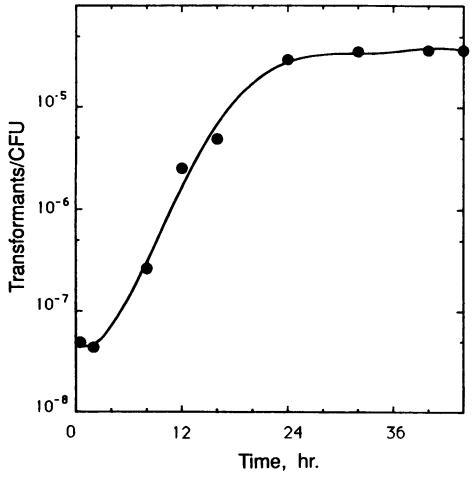

FIG. 2. Expression of rifamicin resistance in transformants. PR-6 cells were transformed with Rif^r DNA by the standard transformation procedure. The final concentration of cells was 4×10^7/ml, and the final DNA concentration was 23 μg/ml.

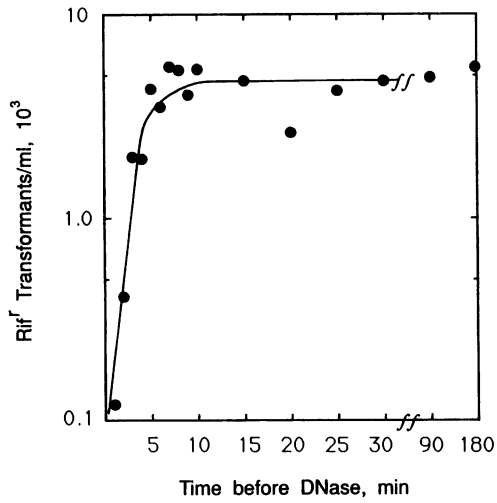

FIG. 3. Kinetics of DNase-resistant DNA binding. PR-6 cells were transformed with Rif^r DNA by the standard transformation procedure. The final concentration of cells was 4×10^7/ml, and the final DNA concentration was 2 μg/ml.

ml; after 22 h of dark preincubation, there were 3.9×10^7 cells per ml.

Phenotypic expression of rifamicin resistance. Stevens and Porter (29) previously established that the phenotypic expression of drug resistance by Str^r DNA-transformed PR-6 required a significant period of incubation before challenge with streptomycin. PR-6 cells were transformed with Rif^r DNA by the standard transformation procedure (the final DNA concentration was 23 μg/ml) and plated. Plated cells were then challenged with 90 μg of rifamicin per plate (3 μg/ml, final concentration) at the indicated times. Cells for CFUs were plated from the reaction mixture at the time of transformation. Resistance to rifamicin in PR-6 transformants increased in a linear fashion with time over the first 20 h (Fig. 2). The response then essentially leveled off, with only a slight increase apparent over the next 24 h.

Kinetics of DNase-resistant DNA binding. The time required to achieve maximal transformation of recipient cells with Rif^r donor DNA was determined (Fig. 3). Transformation with 20 μg of DNA per ml that conferred the Rif^r phenotype was terminated at the indicated times by using DNase I. The standard transformation protocol was followed. After about a 1-min lag, during which no transformants were evident, a rapid linear increase in the number of transformants was observed over the next 4 min. The number of transformants then leveled off at approximately 5.5×10^3/ml, and no significant increase in the number of transformants was subsequently observed.

Dependence of transformation on DNA concentration. To design and interpret DNA competition experiments and eclipse experiments, it is necessary to know whether there is a region of linear relationship between DNA concentration and the number of transformants formed, whether saturation occurs, and whether the markers used in the experiments represent multisite or single-site mutations. This information was previously established for the Str^r mutant of PR-6 (29) and reconfirmed for the study described here. Similar information was generated for the Rif^r mutant through use of the modified transformation protocol. A linear region of increase in transformants per ml versus DNA concentration from about 0.005 μg/ml through approximately 0.1 μg/ml (final concentration) in the transformation mixture was observed (Fig. 4). The DNA concentration dependence leveled off at about 1.0 μg/ml and at a yield of about 1×10^6 transformants per ml or 2×10^{-3} transformants per CFU. Modification of the transformation procedure yielded a significant increase in transformants per milliliter and in transformants per CFU compared with values obtained with the standard transformation procedure (see Materials and Methods), which was used to generate the data shown in Fig. 2 and 3. Compare 1×10^6 transformants per ml or 2×10^{-3} transformants per CFU obtained with the modified transformation procedure (Fig. 4) with 1.5×10^3 and 4.7×10^3 transformants per ml or

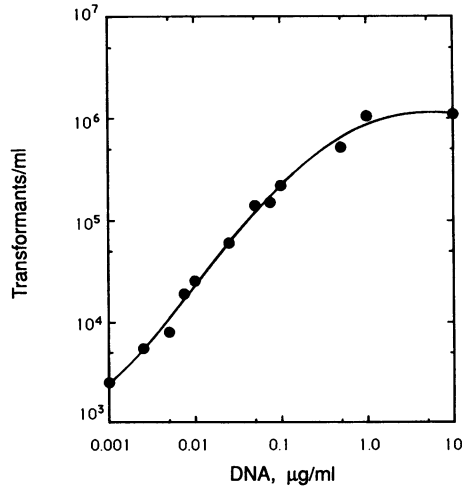

FIG. 4. Dependence of transformation on DNA concentration. Serial dilutions of chromosomal Rif^r DNA were made to yield a range of concentrations from 0.01 to 100 μg/ml, 0.1 ml of an appropriate dilution of DNA was transferred to tubes previously equilibrated to 30°C, and 0.9 ml of PR-6 grown to a density of 5.5×10^8 cells per ml at 39°C was added to each tube. The tubes were incubated for 20 min, without bubbling with CO_2, at 30°C. DNase was added to each tube to a final concentration of 10 μg/ml. Challenge with drug was done by underlaying 90 μg of rifamicin in 2 ml of 0.6% A$^+$ agar.

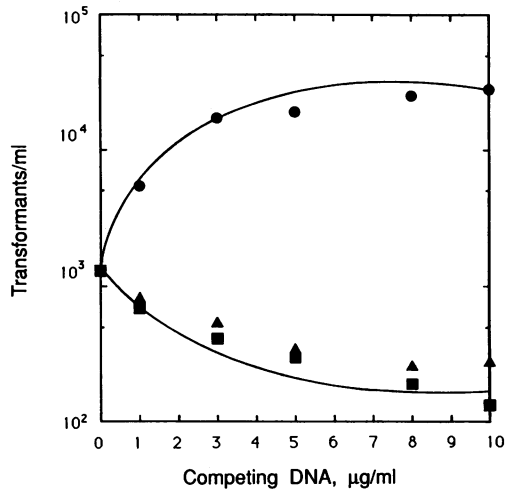

FIG. 5. DNA-DNA competition. Exponentially growing cells of PR-6 at a concentration of 8.9×10^7/ml were transformed by the standard transformation procedure. In the first type of experiment, the final Rif^r DNA concentration was held constant at 2 μg/ml in the transformation reaction mixture while the final Str^r DNA concentration was varied from 0 to 10 μg/ml. Typical results for Rif^r (▲) and Str^r (●) transformants are shown. In the second type of experiment, the final Rif^r DNA concentration was held constant at 2 μg/ml while final DNA concentration from *E. coli* W3110 was varied from 0 to 10 μg/ml. ■, Rif^r transformants. Transformation with DNA from W3110 was not observed (see text). Typical results are shown. DNA uptake was terminated by the addition of DNase at a final concentration of 10 μg/ml.

3.7×10^{-5} and 1.2×10^{-4} transformants per CFU obtained with the standard transformation procedure (Fig. 2 and 3, respectively). The slope of the linear region of the curve shown in Fig. 4 was calculated to be 0.97. Half-saturation of the transformation reaction occurred at approximately 0.5 μg/ml. The transformation efficiency determined from the curve shown in Fig. 4 was about 10^6 transformants per μg of DNA.

DNA binding. Stevens and Porter (30) reported that DNA from members of the cyanobacterial genera *Synechococcus* and *Synechocystis* heterospecifically transformed PR-6. These results suggested that the DNA-binding reaction of PR-6 was nonspecific, at least in regard to cyanobacterial sources of DNA. In an attempt to further evaluate this apparent lack of specificity and to quantitate it, we performed DNA-DNA competition experiments with both homologous and clearly heterologous DNA. In the first experiment, PR-6 Rif^r DNA was competed with homologous PR-6 Str^r DNA. The concentration of Rif^r DNA in the transformation reaction was held constant at 2 μg/ml. The competing DNA was added in increments such that its final concentration in the transformation reaction ranged from 0 to 10 μg/ml. The standard transformation protocol was used. When Rif^r DNA from PR-6 was competed with Str^r DNA from PR-6, a rise in the number of Str^r transformants was seen concurrently with a decline in the number of Rif^r transformants (Fig. 5). As the total DNA concentration was increased, the rate of change in the number of transformants per milliliter decreased in a curvilinear manner. These results were used as the control for the second experiment, in which PR-6 Rif^r DNA was competed with heterologous unmarked DNA from *E. coli* W3110 at the same concentrations used for Str^r DNA in the first experiment. Although it was not possible to select for markers on W3110 DNA, it was apparent that a response analogous to that seen in the first experiment occurred. The decrease in Rif^r transformants closely paralleled that seen when Str^r PR-6 DNA was used as the competing DNA.

TABLE 1. Effects of temperature on transformation[a]

Donor DNA	No. at given transformation temp (°C)		CFU/ml (10^7)	Fold increase
	30	39		
Str^r	1.7×10^4 tfo/ml 5.1×10^{-4} tfo/CFU	3.9×10^3 tfo/ml 1.0×10^{-4} tfo/ml	3.3	5.1
Rif^r	2.6×10^4 tfo/ml 6.4×10^{-4} tfo/CFU	3.4×10^3 tfo/ml 8.6×10^{-5} tfo/cfu	4.0	7.4

[a] PR-6 cells were grown at 39°C. Transformations were done by the standard protocol but at the designated temperature. Rif^r and Str^r DNA was present at a final concentration of 10 μg/ml. Platings for CFU were done at the time of transformation. tfo, Transformants.

DNA uptake and processing. Since several of the experiments described below were directed at elucidating DNA-processing events during transformation (which are known to occur very rapidly), it was of value to know whether these experiments could be conducted at 30°C rather than at the usual 39°C. The results were surprising in that a substantial average increase in transformation frequency of approximately sixfold occurred when transformation was done at 30°C rather than 39°C (Table 1). When cells were grown at 30°C and also transformed at 30°C, similar results were obtained (data not shown).

It was necessary for performance of the eclipse experiment to know whether single-stranded DNA could transform PR-6 cells. Samples of Rif^r DNA were divided into two subsamples. One of the subsamples was heat denatured, rapidly chilled, and immediately used to transform one of two samples of PR-6 cells. The second subsample of Rif^r DNA was left untreated and used to transform the second sample of PR-6 cells. Results of several independent repeats of this experiment showed that undenatured, and presumably double-stranded, Rif^r DNA yielded normal transformation frequencies (Table 2). However, we did not detect any transformants when denatured, and presumably single-stranded, Rif^r DNA was used as the transforming DNA.

DNA eclipse. Having shown that single-stranded DNA does not possess transforming activity, we could now ask whether DNA eclipse occurred. However, before the eclipse experiment was feasible, a rapid lysis procedure for the cells of PR-6 was necessary. The constraints on the procedure

TABLE 2. Transforming activity of denatured Rif^r DNA[a]

Rif^r DNA and trial no.	Transformants/ml (10^4)	CFU (10^7)	Transformants/CFU (10^{-4})
Native			
1	1.7	9.0	1.9
2	1.9	6.8	2.9
3	1.8	5.6	3.2
Denatured			
1	0	9.0	
2	0	6.8	
3	0	5.6	

[a] Rif^r DNA in SSC was heat denatured at 100°C for 10 to 30 min and then quick chilled at −10°C to prevent reannealing of the DNA. Exponentially growing PR-6 cells were then immediately transformed with denatured or native DNA by using the standard transformation protocol. Single-stranded DNA was verified by agarose gel electrophoresis and the change in A_{260}.

TABLE 3. Test of the crude lysate procedure[a]

Trial	Prelysis CFU of resistant cells	Cells in crude lysate		Frequency (transformants/ml)		No. of recipient cells (CFU/ml)	
		Microscopy	CFU/ml	Str^r	Rif^r	Before addition of lysate	After addition of lysate
1	1.0×10^7	None	0	2.6×10^3		6.0×10^7	5.2×10^7
2	2.5×10^8	Few rounded	8	2.7×10^2		2.1×10^8	2.2×10^8
3	4.1×10^7	None	0		1.1×10^4	4.2×10^7	4.6×10^7
4	5.2×10^8	None	2		4.3×10^3	3.1×10^7	3.3×10^7

[a] Either Str^r or Rif^r PR-6 cells were subjected to the crude lysis protocol (see Materials and Methods). After lysis, 0.1 volume of crude lysate was added directly to 0.9 volume of wild-type PR-6. After addition of the crude lysate, the standard protocol for transformation and challenge with the respective drug was followed. Viable counts were performed before and after cells had been added to the crude lysate in the transformation tube. Crude lysates were examined microscopically for morphologically normal cells, and platings were made directly from crude lysates by the usual methods to determine whether significant numbers of resistant cells were surviving the lysis treatment.

were rapidity of execution, the fact that the lysate at a 1:10 dilution had to be of insufficient strength to cause further lysis of cells, and the requirement that the diluted lysate not significantly affect transformation frequency. Use of the crude lysis protocol given in Materials and Methods and multiple trials with both Str^r and Rif^r PR-6 cells as donors and wild-type cells of PR-6 as recipients yielded acceptable results (Table 3). Str^r and Rif^r transformants were observed at frequencies of 2.7×10^2 to 1×10^4/ml. In addition, viable cell counts at the time of transformation were not significantly affected, whereas essentially none of the cells in the initial crude lysates survived.

The DNA eclipse experiment could now be performed as follows. Rif^r PR-6 cells were transformed by using purified Str^r DNA. A 4-min period was allowed for binding and uptake of DNA. The reaction was then terminated by addition of DNase to a final concentration of 10 μg/ml. After 30 s, the first sample was removed and promptly added to a 10-fold volume of SSC at −5 to −10°C to stop DNA processing in the recipient cells. Subsequent samples were removed and likewise chilled at the indicated time points. All subsequent processing was performed at 0°C until quick lysis. The samples of recipient cells were then subjected to quick lysis, and the crude lysate was frozen until used for the second-step transformation. In the second step, 1 volume of thawed crude lysate was added to 9 volumes of wild-type PR-6 cells. The modified transformation protocol was then used to enhance transformation frequencies, and duplicate platings were done. After 40 h, half of the plates were challenged with rifamicin. Counts of transformant colonies from these plates were used as an internal control for lysis efficiency and the vagaries of experimental handling; the other half were challenged with streptomycin. Transformants to each of the drug resistance phenotypes were scored, and the ratio of the incoming Str^r marker to the resident Rif^r marker was determined. This ratio was consistently in the range of 10^1 Str^r colonies to 10^4 Rif^r colonies. The data were normalized to the highest Str^r/Rif^r ratio obtained in a given experiment. The normalized results of five experiments were then averaged. Even at the earliest sampling times, there was no detectable loss in the transformation frequency of the Str^r marker, and variation throughout the experiments showed no more than a 25% fluctuation from the mean (Fig. 6). No early rise period was evident, nor did the ratios change even at times well over 60 min after exposure to DNA had been terminated.

DISCUSSION

Attempts to further define physiological competence reinforced the notion that PR-6 was broadly competent during exponential growth and began to lose competence only upon entrance into the stationary phase of growth. The absence of light before exposure of cells to exogenous DNA resulted in a marked decline in transformation frequency. These observations taken together suggest that the energy charge of the cell and its competence for transformation are related. The use of pronase E had no effect on the competence of PR-6 for transformation, suggesting that the presence of a soluble protein competence factor or factors is unlikely. Similar results have been observed for transformation in *Neisseria gonorrhoeae* (2), in which a competence peak is also lacking.

Large differences in phenotypic expression for ampicillin versus kanamycin in PR-6 and for ampicillin versus chloramphenicol in *Anacystis nidulans* R2 have been observed (4, 22). To study DNA-DNA competition and especially DNA eclipse, it was necessary to have genetic markers that behaved similarly under experimental conditions. It was particularly important that they behave as single markers. There was virtually no difference in phenotypic expression or the time dependence of transformation between Str^r and Rif^r in PR-6 (see reference 29 for comparison). The direct relationship between the frequency of transformation and the concentration of donor DNA (slope = 0.97 in Fig. 4) indicates that a single collision between donor DNA and a recipient cell results in a transformant. Single-hit kinetics for both chromosomal (13, 22, 25) and chimeric plasmid (6) transformation have been previously reported. Thus, there is

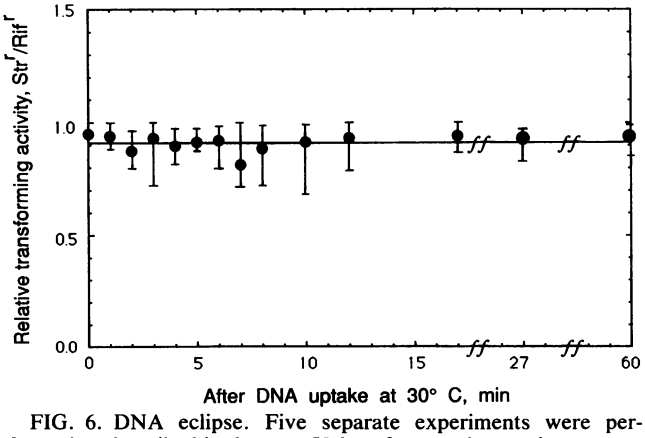

FIG. 6. DNA eclipse. Five separate experiments were performed as described in the text. Values from each experiment were normalized, and the normalized values were averaged as described in the text. An early rise period from very low values to a relatively constant maximum was not observed. Values remained essentially constant from as early as 4 min after initial exposure to DNA, which represents the zero time point of reaction termination. Vertical bars represent 1 standard deviation from the mean results of five experiments.

now considerable evidence supporting the notion of a single DNA-binding event.

A modest change in medium A allowed for a modest increase in cell density, which resulted in more transformants per milliliter. Dropping the temperature at which transformations were done from 39 to 30°C resulted in a further increase in transformants per milliliter and, perhaps more significantly, in transformants per CFU. These two changes were essential for the eclipse experiment but also provided a practical advantage for future chromosomal transformation experiments.

DNA eclipse is a term used to describe the temporary loss of transforming activity that occurs when donor DNA is immediately converted to a single-stranded state during uptake (16). This temporary loss of donor DNA activity is due to the inability of the single-stranded DNA generated during passage through the cell envelope to be bound and taken up by other competent cells until it is restored to the double-stranded state by recombination with the recipient cell chromosome. DNA eclipse is observed in the gram-positive organisms *Streptococcus pneumoniae* and *Bacillus subtilis* (10, 11, 35), in which it has been demonstrated that the internalized donor DNA is single stranded after uptake (14, 21). This phenomenon is not observed, however, in the gram-negative organisms *Haemophilus influenzae* and *N. gonorrhoeae* (3, 31, 36). In the case of *H. influenzae*, it has been shown that double-stranded donor DNA is first taken into membrane-bound vesicles called transformasomes, where it is resistant to externally added DNase (17, 18). Although the movement of the donor DNA from the *H. influenzae* transformasome to the cytoplasm is likely to involve conversion to single strandedness (1), the donor DNA recovered from the transformasomes during DNA eclipse experiments retains its transforming activity.

Results of our work with PR-6 indicate that single-stranded DNA lacks transforming activity. Thus, if a marker introduced into these cells by means of transformation did undergo a transition to a single-stranded intermediate, one would expect this to be reflected by a temporary loss of transforming activity. In the pneumococci, recovery from eclipse for a single-site marker is complete about 10 min after the initial loss of activity (11, 14). In PR-6, no loss of donor DNA activity with respect to the resident Rif^r marker was observed from the earliest possible sampling times until well over 1 h after the cells were transformed. Because of the extended time period before PR-6 transformants can be selected (Fig. 2), it might be argued that we have simply failed to detect eclipse because the relevant DNA processing events are spread out over a long time period. We feel that this delay in the appearance of the transformed phenotype is more likely to be a matter of slow expression, however, since DNA processing through the uptake stage is quite rapid (Fig. 3). The probable absence of eclipse in PR-6 suggests that the postuptake processing of transforming DNA in this organism is more similar to that of *H. influenzae* and *N. gonorrhoeae* and less similar to that of the pneumococci and *B. subtilis*. However, we lack any evidence for the transformasome that has been implicated in the postuptake processing of transforming DNA in *H. influenzae* (1, 17, 18).

The gram-positive heterotrophs, particularly the pneumococci and *Bacillus* spp., have been shown to bind both heterologous and homologous DNA (15, 26). In contrast, *H. influenzae*, a gram-negative heterotroph, preferentially binds only homologous DNA (24) through recognition of an 11-base-pair sequence in homologous DNA (7) that is lacking in heterologous sources of DNA. *N. gonorrhoeae*, another gram-negative heterotroph, also demonstrates DNA uptake specificity (9). The DNA-binding reaction in PR-6 is clearly nonspecific, since heterologous DNA from *E. coli* competed equally with homologous DNA for the binding sites available on PR-6 cells. In this regard, PR-6, which has a gram-negative outer envelope, appears to be similar to the transformable gram-positive bacteria. PR-6 is therefore unique among the physiologically competent organisms thus far characterized in that it combines lack of specificity in DNA uptake with the lack of DNA eclipse.

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