

Release and Uptake of Gene Transfer Agent by *Rhodopseudomonas capsulata*

MARC SOLIOZ, HUEI-CHE YEN, AND BARRY MARRS*

Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri 63104

Received for publication 4 March 1975

Many strains of *Rhodopseudomonas capsulata* are capable of exchanging genetic information via a recently discovered gene transfer process involving the release and subsequent uptake from the medium of particles containing genetic information (gene transfer agents, GTAs). No viral activities are observed to be associated with this system. An assay has been developed to quantitate gene transfer in *R. capsulata*. Conditions are described for which the number of cells acquiring a new genetic trait is directly proportional to the number of GTAs and independent of the number of recipient cells. These conditions were used for the assay of the uptake and release of GTAs by cells. The maximum fraction of recipients that acquire a given genetic marker is $\sim 4 \times 10^{-4}$. Free GTA appears in a growing culture in one or two abrupt waves near the time of transition from exponential to stationary phase. During these waves, the titer of GTA for a given marker may reach 2×10^6 /ml. A comparison of the frequency of single- and double-marker transfers suggests that most of the cells in early-stationary-phase cultures are active recipients. The ultraviolet inactivation spectrum of GTA resembles that of the small ribonucleic acid phages. The inactivation cross section σ , for GTA was calculated to be 1.7×10^{-16} cm²/photon at 265 nm.

We have recently described the discovery of the first genetic exchange system for a nonsulfur purple photosynthetic bacterium (3). Certain strains of *Rhodopseudomonas capsulata* are capable of releasing particles that contain samples of genetic information representing all parts of the genome. These particles can subsequently provide genetic information to other bacteria of the same species, thus effecting genetic transfers. The process resembles transduction; however, the particles are much smaller than any known transducing bacteriophage. We propose to call these particles gene transfer agents (GTAs), and cells that express new genetic markers received via GTAs are termed transferants. It is our goal to be able to use this genetic system to study energy metabolism and the regulation of photosynthetic membrane formation via a biochemical genetics approach. This report describes the development of a quantitative assay for *R. capsulata* gene transfer, the use of the assay to study some of the characteristics of the system, and further evidence about the physicochemical nature of the vector mediating the gene transfer.

MATERIALS AND METHODS

Bacterial strains. *R. capsulata* B10 and B6 have been described previously (3). The following strains

were derived from B10: BB101 (carries marker *rif-10* which confers resistance to rifampin [Rif^R]); BB102 (carries marker *str-1* which confers resistance to streptomycin [Str^R]); BB1012 (carries *rif-10* and *str-1*); YB1020 (carries *str-2* which confers streptomycin resistance and *trpA20* which causes tryptophan auxotrophy [Trp⁻]).

Media. Peptone-yeast extract medium (PYE; 0.3% peptone [Difco] and 0.3% yeast extract [Difco] in deionized water) was used for growth of recipient and donor cultures. For strain YB1020 this was supplemented with 10 μ g of L-tryptophan per ml. RCV, a minimal salts medium (5), supplemented with 0.3 μ g of L-tryptophan per ml was used as a selective medium to detect tryptophan-independent cells.

Gene transfer assay. Recipient cells were harvested from early-stationary-phase photosynthetic cultures by centrifugation and then suspended in buffer containing 1 mM MgCl₂, 1 mM CaCl₂, 1 mM NaCl, and 10 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.8) (G buffer) supplemented with 500 μ g of bovine serum albumin (Sigma Chemical Co., fraction V) per ml. Recipients were routinely harvested shortly before use, but occasionally were stored on ice for several hours without serious loss of activity. GTA was prepared routinely by filtering a donor culture through a 0.45- μ m membrane filter, since removing cells by low-speed centrifugation results in a 50% loss in gene transfer activity compared to membrane filtration of the same culture. The resulting cell-free filtrate may be used directly or purified further to remove inhibitory substances. GTA may be

partially purified and concentrated by diafiltration against G buffer plus 500 μg of bovine serum albumin in an amicon thin-channel filtration apparatus fitted with an XM300 membrane. Diafiltered concentrates may be stored at -70 C provided bovine serum albumin is present.

Recipient cells and donor filtrate were mixed in G buffer and incubated aerobically at 35 C for about 60 min. During this incubation, the process of gene transfer is sensitive to inhibition by a wide variety of substances, including peptone and yeast extract, so the incubation mixture is constituted to minimize carry-over of residual medium from donor and recipient cultures. After this incubation, recipients were diluted, plated, and incubated at 35 C under nonselective conditions to allow phenotypic expression of newly acquired genetic traits. Different genetic markers require different times for phenotypic expression. Rifampin resistance gene transfers were titered by plating recipients in a molten, soft-agar layer on PYE plates and incubating for 4 h at 35 C before overlaying with molten agar containing sufficient rifampin to bring the final concentration to 67 $\mu\text{g}/\text{ml}$. Prototrophic transferants were selected from tryptophan auxotrophs by plating directly in RCV agar supplemented with 0.3 μg of L-tryptophan per ml. This level of tryptophan allows phenotypic expression but limits the growth of the parental auxotrophs to microcolonies.

UV light irradiation. The stocks of bacteriophage T2 and GTA used throughout the experiments had titers of 5×10^{11} plaque-forming units per ml and 2×10^6 rifampin resistance gene transfer units per ml, respectively. The GTA stock was prepared by concentration and diafiltration as described above. All dilutions were done with G buffer. T2 and GTA stocks were diluted and mixed to give a titer of about 10^6 plaque-forming units per ml for T2 and 2×10^4 resistance gene transfer units per ml for GTA. Portions (1 ml each) of this mixture were irradiated in a quartz cuvette (1 by 1 by 4 cm) with magnetic stirring. As a source for monochromatic light, we used a spectrofluorometer (Aminco-Bowman) equipped with a high-pressure xenon lamp. A dose of 100 ergs/ mm^2 at 260 nm was delivered in approximately 200 s. The irradiated samples were assayed for the remaining GTA gene transfer activity as described above. T2 plaque-forming ability was measured by plating with *E. coli* S. All handling of ultraviolet (UV)-irradiated samples was done in red light to avoid possible photoreactivation.

RESULTS

Assay conditions. Two criteria were used in developing the assay. First, conditions should be found for which the number of transferants is directly proportional to the amount of input GTA and invariant with the number of potential recipient cells present. Second, conditions should be optimized so that a fixed number of GTAs gives rise to a maximum number of transferants. The assay conditions described in Materials and Methods meet the first of these

goals as demonstrated by the results shown in Fig. 1 and 2. When a constant number of recipient bacteria are exposed to increasing numbers of GTAs (Fig. 1), the resulting number of transferants increases proportionately until about 4×10^{-5} of the cells have expressed a given gene transfer. Further increases in GTA per recipient result in a nonlinear increase in transferants until the frequency of transferants reaches a maximum at about 3×10^{-4} to 4×10^{-4} . This saturation occurs at GTA/recipient ratios on the order of 100 times greater than the maximum ratio giving a linear response.

The values on the linear portion of the dose-response curve may be used to calculate a "titer" of the number of *rif-10* GTA per ml of solution; however, since the overall efficiency of gene transfer is not yet known, this titer represents only assayable *rif-10* transfer units. Furthermore, since the titer is measured for only one genetic determinant, the total number of GTA, is greater than this titer by at least a factor equal to the molecular weight of the genome divided by the average GTA equivalent molecular weight. We estimate this factor to be large (10^3 to 10^4) because a 70S particle (3) with a buoyant density of $\sim 1.37\text{ g}/\text{cm}^3$ in CsCl (un-

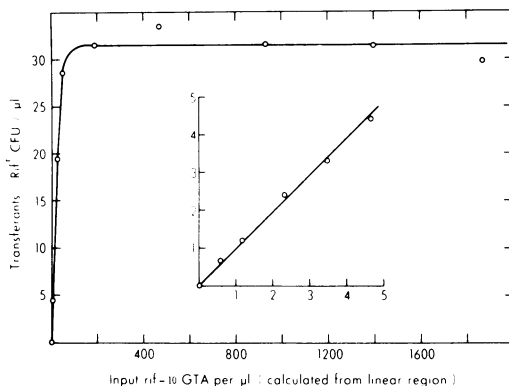


FIG. 1. Number of rifampin resistance gene transfer events among a constant number of recipients treated with various amounts of GTA. Rifampin-sensitive recipient cells (B6) were grown, harvested, and suspended at a final density of 9×10^7 colony-forming units (CFU) per ml in G buffer plus 500 μg of bovine serum albumin per ml containing various dilutions of a concentrated, diafiltered preparation of GTA from strain BB101. The mixture of cells and GTA was incubated for 2 h at 35 C and then diluted and plated for determination of rifampin resistance. The background frequency of spontaneous rifampin resistance in this experiment was less than 4.0 CFU/ml. The input titer of GTA is calculated assuming one Rif^R CFU arises from one *rif-10* GTA in the region where the dose-response curve is linear, and is an "assayable" titer only. The inset is an expansion of the initial portion of the curve.

published data) would be expected to carry a nucleic acid molecule of relatively small size ($\sim 10^5$ to 10^6 daltons).

Above a critical ratio of recipients per GTA, the assay is insensitive to variations in the number of recipients (Fig. 2). At higher recipient cell concentrations, O_2 becomes limiting and a gradual decrease in transferants is observed (data not shown) unless steps are taken to improve aeration. We have also observed that 1 mM KCN strongly inhibits the gene transfer process (data not shown), and these two results taken together suggest that this process requires energy metabolism during the initial stages.

Our success in meeting the second assay criterion, that of maximal response, cannot be judged until more of the molecular details of the system are known. We have examined the effects of ionic composition and concentration (and pH) on both the stability of GTAs and the assay system itself, and the conditions described above seem optimal. We have been impressed by the wide variety of normally innocuous agents that inhibit the system; e.g., if either 50 mM NaCl or 0.3% peptone is added to the complete assay system, a marked inhibition of transferant production is observed (see below).

GTA uptake. Figure 3 shows the results of an experiment in which the rate of appearance of potential transferants is compared to the disappearance of free, assayable GTA in our standard assay mixture. Potential transferants are measured as the number of cells that are sufficiently committed to gene transfer so that dilution and plating in G buffer soft agar does not interrupt the process. A small correction has been applied to eliminate those gene transfers actually initiated in the soft agar after plating. Since the kinetics of disappearance of free, assayable GTA complements the appearance of potential transferants, we tentatively identify the disappearance of GTAs with their uptake by recipients. The continued rise in transferant titer after all GTA has been taken up (about 30 min) is paralleled by an increase in total cell number and may represent the replication of newly acquired genetic markers. If diluted samples from an assay mix are plated directly into PYE-soft agar, we observe the previously mentioned inhibitory effect of PYE. The separation of these two transferant appearance curves suggests that the PYE-sensitive phase is over about 10 min after uptake of GTA.

Active recipient population. Analysis of the frequency of double gene transfers (Table 1) is interpreted to indicate that nearly all of the cells in the population are active recipients in

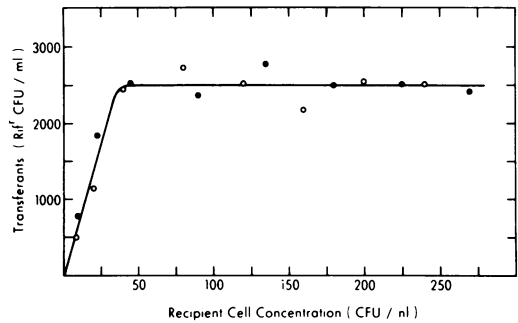


FIG. 2. Number of rifampin resistance gene transfer events induced by a constant amount of GTA in various numbers of recipient bacteria. The gene transfer assays were performed as described in the legend to Fig. 1, except that each assay contained the same amount of GTA and various amounts of a washed recipient cell suspension. The same amount of the same preparation of GTA (stored at -70°C) was assayed on two different days using different cultures of recipients: assay 1, ●; assay 2, ○. CFU, Colony-forming units.

our assay. If we assume that the gene transfers for rifampin resistance and tryptophan independence are independent events, i.e., no genetic linkage between these markers and no exclusion of a second gene transfer by a first, then we can compare the observed frequency of double events (9.6×10^{-9}) to the expected frequency calculated as the product of the frequencies of the single events (7.2×10^{-9}). We consider this agreement to be excellent and to indicate that total cell number is a good estimate of the number of active recipients in the assay.

GTA production kinetics. To determine when during the growth cycle GTA was released, samples of cultures were filtered and assayed for GTA at various times after inoculation of photosynthetic cultures of strain BB101 or BB1012. The surprising result is shown in Fig. 4, which summarizes the data from four independent trials. Free GTA concentration increases abruptly in filtrates shortly before the transition from log to stationary phase. However, before stationary phase is reached, GTA titer decreases as abruptly as it appeared, only to reappear at even higher titers as turbidity becomes constant. Occasionally the second wave of free GTA does not materialize, but the peak of GTA titer during the transition from exponential growth to stationary phase has been observed in all trials to date. The sharp drop in GTA titer at about 16 h might be due to a short, intense period of GTA uptake by the producing culture. To examine this possibility, we removed cell samples from cultures during this

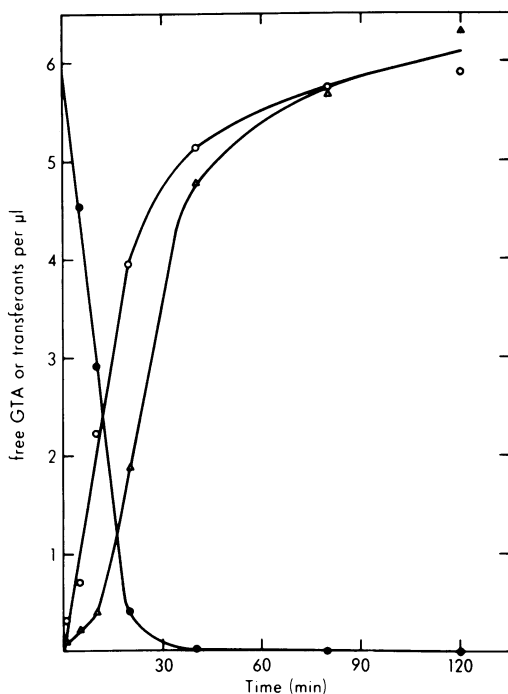


FIG. 3. Kinetics of GTA uptake and appearance of potential transferants. Three flasks of G buffer plus 500 μg of bovine serum albumin per ml were incubated at 35 C with gyratory mixing. At zero time, flask I received recipient cells (strain B6) and GTA (diafiltered; from strain BB101), flask II received GTA alone, and flask III received cells alone. The zero-time cell concentration in I and III was 4.7×10^8 colony-forming units per ml. The input GTA titer in I and II was 5.7×10^3 rif-10 GTA per ml. At various times, samples were removed and assayed for unadsorbed GTA and cells committed to become rifampin-resistant transferants. Unadsorbed GTA were measured by filtering the cell-GTA mix on 0.45- μm cellulose acetate membrane filters and assaying the filtrate for GTA (\bullet). Potential transferants at various times were measured by diluting a sample from flask I 10-fold in G buffer plus 500 μg of bovine serum albumin per ml and then 12.5-fold into G buffer plus 0.6% agar (molten) in an empty petri plate. These plates were incubated at 35 C (after the agar gelled) until 2 h had elapsed from the initial mixing of cells and GTA, at which time all plates were overlaid with 10 ml of 0.3% PYE soft agar and incubation continued for 4 h at 35 C. Then PYE-soft agar containing rifampin was poured over the plates as usual, and the plates were incubated until rifampin-resistant colonies formed. Dilution and immobilization in agar were intended to prevent further association between cells and GTA. To test the effectiveness of this procedure, small volumes were removed from flasks II and III, similarly diluted, mixed on empty petri plates with the addition of 5.0 ml of G buffer-soft agar, and then treated exactly as above. Transferants arising from plate adsorption were most numerous at

period and tested the cells for their ability to bind GTA. Cells were spun down, suspended at a constant turbidity, and exposed to a known saturating titer of genetically marked GTA. After sufficient time for complete adsorption, mixtures were filtered and free GTAs were titered. This experiment (data not shown) indicated that cells from cultures in transition stage had the same adsorption activity as early-stationary-phase cells. It would thus seem that fluctuations in the rate of GTA uptake do not account for the observed fluctuation in free GTA titer. Furthermore, strain H9, which produces but does not adsorb GTA under the conditions described in Fig. 3, shows fluctua-

TABLE 1. Frequency of gene transfer

New phenotypes ^a	CFU/ml ^b	Frequency ^c
Single events, GTA induced		
Rif ^S \rightarrow Rif ^R	1.1×10^5	1.0×10^{-4}
Trp ⁻ \rightarrow Trp ⁺	8.0×10^4	7.2×10^{-5}
Single events, spontaneous ^d		
Rif ^S \rightarrow Rif ^R	2.5×10^1	2.3×10^{-8}
Trp ⁻ \rightarrow Trp ⁺	8.7×10^2	7.9×10^{-7}
Double events, GTA induced, observed		
Rif ^S , Trp ⁻ \rightarrow Rif ^R , Trp ⁺	$1.06 \times 10^{1*}$	9.6×10^{-9}
Double events, GTA induced, calculated ^f		
Rif ^S , Trp ⁻ \rightarrow Rif ^R , Trp ⁺	7.9	7.2×10^{-9}
Double events, spontaneous, observed ^d		
Rif ^S , Trp ⁻ \rightarrow Rif ^R , Trp ⁺	0 ^g	$<7 \times 10^{-10}$

^a YB1020 cells, 1.1×10^9 /ml (optical count), were treated with filtrate from BB101 containing 2×10^8 rif-10 GTA per ml, and transferants were selected.

^b CFU, Colony-forming units.

^c Frequency of particular new phenotype obtained by dividing concentration of new-type cells by total cell concentration.

^d No GTA added.

^e 106 CFU observed.

^f Expected frequency calculated as product of single-event frequencies; CFU per milliliter calculated as: expected frequency times total cells per milliliter.

^g No CFU observed per 1.4×10^8 recipients.

early times (650/ml at 1 min) and decreased gradually as incubation continued. The difference between the number of Rif^R colony-forming units on these two types of plates was plotted as transferants per milliliter (\circ). Samples from flask I were also removed, diluted, plated directly in PYE-soft agar (instead of G buffer-soft agar), and then treated as described for the previous plates. The numbers of Rif^R colony-forming units appearing on these plates are also plotted (Δ).

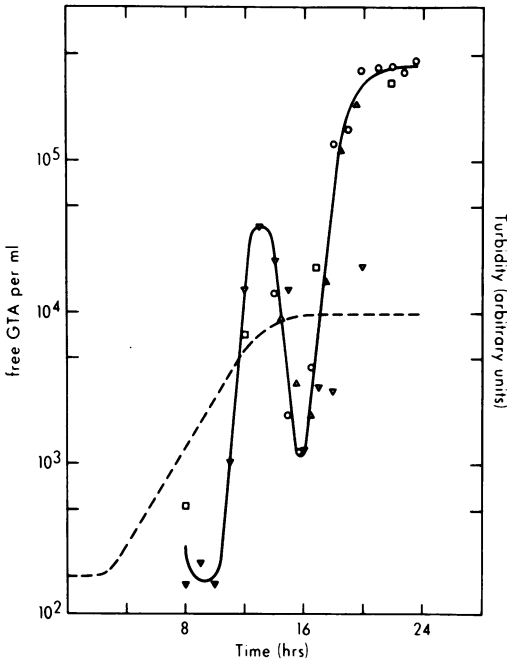


FIG. 4. Time course of GTA release. Samples of photosynthetically growing cultures of strains BB101 or BB1012 were diluted 1:3 in G buffer plus 500 μ g of bovine serum albumin per ml, filtered through 0.45- μ m cellulose acetate membranes, and frozen at -70 C. The following day, samples were thawed and assayed for either rif-10 (O, □, Δ) or str-1 (∇) GTA. BB101 cultures (O, □, Δ) were grown in screw-cap culture tubes, two tubes being harvested for each time point. Strain BB1012 (∇) was grown in a plastic syringe, and samples were removed periodically by depressing the plunger. Culture growth was followed by turbidity measurements, which are plotted relative to the stationary-phase turbidity. Time scales were then normalized so that 50% maximal turbidity occurred at a fixed time. The resulting growth curves were essentially superimposable and are depicted by -----.

tions in free GTA titer similar to those shown in Fig. 4. The possibility remains that conditions of GTA uptake in the culture are not preserved in our uptake assay and, therefore, uptake may still account for the unusual changes in titer.

A second possible explanation for the drop in GTA titer at about 16 h is that an inhibitor of our assay might be produced and later destroyed. This has been ruled out by a simple mixing experiment which showed that exogenous, genetically distinct GTA, when mixed with filtrates of samples from a growing culture, retain a constant titer, whereas the endogenous genetic marker fluctuates as shown in Fig. 4. This leaves open the possibility of a labile inactivating factor which destroys GTA in the

producing culture but is not active in the in vitro mixture.

Inactivation of GTA by UV light. The rate of inactivation of gene transfer activity of GTA by UV light was determined. Since such measurements depend strongly on the intensity of the light source used, the extent of internal absorption and light scattering of the sample, and the sample geometry, we calibrated our system with bacteriophage T2, which has a known UV sensitivity. Identical irradiation conditions were assured by using a mixture of T2 and GTA throughout the experiments. Figure 5 depicts inactivation curves at 260 nm. Appropriate control experiments showed that T2 and GTA did not interfere with each other in either the assays or the irradiations. The experimental curves can be represented by the equation $n = n_0 \exp - \sigma D$, where n_0 is the initial titer and n is the titer after a dose (D) is delivered; the inactivation cross section, σ , is a constant which is proportional to the sensitivity of a particular biological material (e.g., virus) at a given wavelength and corresponds to the slope in Fig. 5. The dose was calculated from the σ value for T2 as determined by Rauth (6). The inactivation of GTA was found to be exponential over at least three logs.

Action spectrum. In Fig. 6, the inactivation cross sections for GTA and T2 are plotted as a function of wavelength. The inactivation spectrum of GTA shows a broad maximum at 265 nm and is very similar to that of T2 and other viruses, suggesting that nucleic acid is the target of the UV inactivation. The value of σ for

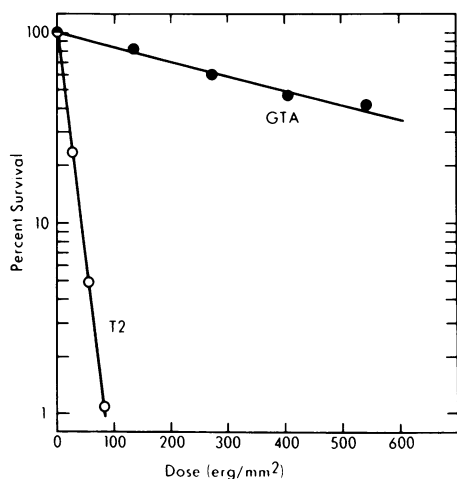


FIG. 5. UV inactivation of bacteriophage T2 and GTA at 260 nm. Symbols: O, percent survival of T2 plaque-forming units; ●, percent survival of rifampin resistance gene transfer activity.

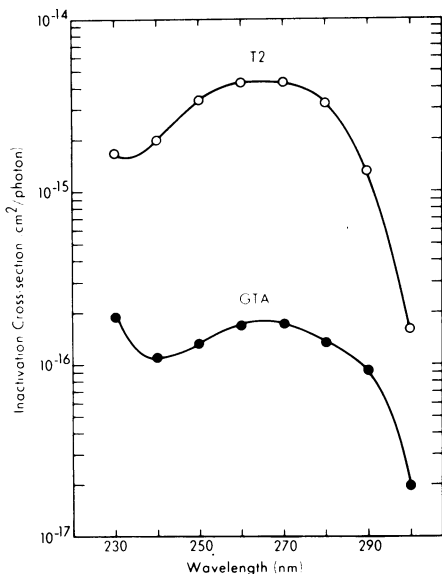


FIG. 6. Action spectrum of inactivation of the plaque-forming ability of bacteriophage T2 (O) and rifampin resistance gene transfer activity (●).

T2 at 265 nm is about 42×10^{-16} cm²/photon; that for GTA is about 1.7×10^{-16} cm²/photon. The ability of GTA to transfer genes is thus approximately 25 times more resistant to inactivation by UV light of that wavelength than the plaque-forming ability of T2. Of the phages for which has been determined (6), the small ribonucleic acid phages MS2, R-17, fr, and 7-S most closely resemble GTA, each exhibiting an inactivation cross section of about 1.2×10^{-16} cm²/photon, whereas, among the deoxyribonucleic acid phages, Φ X174 and fd, with σ values of approximately 10×10^{-16} cm²/photon, are most similar to GTA in their UV sensitivities.

DISCUSSION

The quantitative gene transfer assays described here have permitted the study of production, uptake, and UV inactivation of GTA and have helped define conditions for maximal efficiency of genetic exchange with this system. The high frequency of gene transfer per recipient ($\sim 4 \times 10^{-4}$ for a single marker with excess GTA) compares favorably with most generalized transduction systems and is high enough to be used easily for strain construction and genetic analysis. The observation that gene transfer recipients are saturated when only a small fraction of the cells has acquired a particular new marker may reflect an inherent low efficiency for incorporation of incoming genetic material or it may be due to a limited number of

adsorption sites for GTA on recipient cell surfaces. The analysis of double gene transfer events makes it seem unlikely that a small active fraction of the recipients accounts for all the genetic exchange.

The unusually abrupt appearance and disappearance of GTA in growing cultures are intriguing. It does not resemble the phage production seen in either lysogenic cultures (2) or infected cultures, which release small phage continuously (4). If it is assumed that each particle is relatively efficient in gene transfer and thus the titer by bioassay of a particular genetic marker is a good estimate of the number of genome equivalents released, it is possible that the lysis of only 10^5 to 10^6 cells per ml may account for all the observed GTA production, and thus some sort of lytic phenomenon cannot be ruled out as the mechanism for release of GTA to the medium, since lysis of this small fraction would not have been detected. Alternatively, living cells may shed GTA into the medium without lysing. Release by either mechanism clearly involves a significant degree of synchrony which remains unexplained.

The rate of inactivation of GTA activity by UV light is comparable to that observed for transducing activity (1), but transducing activity usually shows an activation by low doses of UV light which is not observed with GTA. The UV inactivation spectrum of GTA is similar to that for bacterial viruses. Rauth (6) noticed an empirical correlation between the shapes of UV inactivation spectra and strandedness of nucleic acids carried by viruses, namely, the 265/225 ratio for σ values was 2 for double-stranded and 1 for single-stranded nucleic acids with very few exceptions. The GTA spectrum resembles the single-stranded class. Work on the direct characterization of the nucleic acid of GTA is in progress.

The gene transfer system of *R. capsulata* would seem to be either generalized transduction by a defective virus of a size class hitherto not known to transduce or a bacterial sex mechanism unrelated to viral activities. The definitive resolution of these two possibilities will probably be difficult, but in the absence of evidence for viral involvement we favor considering this system as a bacterial mechanism that exists because of the selective advantage which the capability for genetic exchange might confer. We have discovered recently that GTA can be used to demonstrate genetic linkage between the genes for carotenoid biosynthesis and those for bacteriochlorophyll biosynthesis, and mapping of this important region of the *R. capsulata* genome is in progress.

ACKNOWLEDGMENTS

We thank Sandra Bilyeu for her excellent technical assistance, N. Melechen, J. Wall, P. Weaver, and H. Gest for many helpful and stimulating discussions.

This work was supported by Public Health Service grant GM 20173-01 from the National Institute of General Medical Sciences and by grant GB-40359 from the National Science Foundation.

LITERATURE CITED

1. Benzinger, R., and P. E. Hartman. 1962. Effects of ultraviolet light on transducing phage P22. *Virology* **18**:614-626.
2. Jacob, F., and E. L. Wollman. 1959. Lysogeny, p. 319-351. In F. M. Burnet and W. M. Stanley (ed.), *The viruses*, vol. 2. Academic Press Inc., New York.
3. Marrs, B. L. 1974. Genetic recombination in *Rhodospseudomonas capsulata*. *Proc. Natl. Acad. Sci. U.S.A.* **71**:971-973.
4. Marvin, D. A., and B. Hohn. 1969. Filamentous bacterial viruses. *Bacteriol. Rev.* **33**:172-209.
5. Ormerod, J. G., K. S. Ormerod, and H. Gest. 1961. Light-dependent utilization of organic compounds and photoproduction of molecular hydrogen by photosynthetic bacteria; relationships with nitrogen metabolism. *Arch. Biochem. Biophys.* **94**:449-463.
6. Rauth, A. M. 1965. The physical state of viral nucleic acid and the sensitivity of viruses to ultraviolet light. *Biophys. J.* **5**:257-273.