JOURNAL OF BACTERIOLOGY, Jan. 1971, p. 77-85

Vol. 105, No. 1
Printed in U.S.A.

Park-Williams Number 8 Strain of *Corynebacterium diphtheriae*

THEODORE LAMPIDIS[1] AND LANE BARKSDALE

New York University School of Medicine and Medical Center, New York, New York 10016

Received for publication 21 August 1970

Five clones of the Park-Williams number 8 strain of *Corynebacterium diphtheriae*, previously maintained in separate laboratories, were examined for their colonial and biochemical properties, for the restriction and modification system which operates to obscure their lysogeny, and for their capacity to produce large amounts of toxin under ordinary laboratory conditions. The phenotypes of their phage, P, produced in strain 603 and C7 (P·603 and P·C7) differ both as to stability to storage in the cold and to inactivation by antiphage serum. Evidence for a high degree of stability in the integration of P prophage in the PW8 genome is presented.

In 1896 Park and Williams (28) isolated a strain of *Corynebacterium diphtheriae* which had the capacity to produce large amounts of diphtherial toxin. This unusual diphtheria bacillus, the PW8 strain, is of particular importance because it has for 73 years served as the universal source of toxin used for preparing diphtherial toxoid, the agent employed for the immunization of persons against diphtheria. In 1954 it was realized that the PW8 strain carried a bacteriophage (7) later designated P^{tox+} (4). After exposure to inducing doses of ultraviolet light, the Park-Williams 8 strain liberated a small number of phage particles (about 1 plaque-forming unit/10^6 bacteria) capable of forming plaques on the indicator strain, C7 (6, 26). Barksdale interpreted this as indicative of a state of defective lysogeny and designated the rough strain of PW8 with which he worked $PW8_r$ (Pdi), indicating that the P prophage carried by PW8, while inducible, was defective and failed to give rise to infectious particles save in the case of a rare back mutant (7). Others attributed the low phage yields obtained from irradiated PW8 bacilli to its "incomplete electron transport system" (26). Recently, Maximescu (24), employing as an indicator strain *C. ulcerans* strain 9304, tested for the presence of phage in "drops" of "original lysates" of overnight cultures of the Weissensee G strain of PW8 and reported finding about 50 "small, punctiform plaques." Following this lead and employing a strain of *C. ulcerans* 603, we have uncovered a restriction-modification system (8, 14, 22) operating on certain phages grown in strain 603 or PW8 and subsequently plated on strain C7 (Lampidis and Barksdale, Bacteriol. Proc., p. 202, 1970.

[1] Present address: Department of Microbiology, University of Miami School of Medicine, Miami, Fla.

Several laboratories concerned with the production of diphtherial toxoid have become interested in whether or not their strains of PW8 do carry a tox^+ prophage. The investigators in some of these laboratories communicated to us their inability to demonstrate the presence of bacteriophage in the strains of PW8 maintained by them. In one case, sublines of the PW8 strain were reported as failing to produce toxin (29). It might be expected that various substrains of PW8 in different laboratories and under a variety of conditions would in time differ. Up to now, no set of criteria has been established by which strains of PW8 might be compared. In this paper we set down simple procedures for establishing the biochemical properties, toxinogenic capacity, colonial morphology, lysogenic status, and restriction-modification relations of the PW8 bacillus. By using these procedures, the essential features of strains from the United States, Czechoslovakia, England, Holland, and India have been examined.

MATERIALS AND METHODS

Bacteria. Substrains of *C. diphtheria* PW8 were used. CN2000 was obtained through the kindness of D. C. Edwards, the Wellcome Research Laboratories, Beckingham, Kent, England. It has been the basis of a number of studies now fundamental to understanding the corynebacteria. Under suitable conditions it produces large amounts of toxin (11). It possesses a complete cytochrome system (35) and is a much smoother strain than $PW8_r(P)^{tox+}$ and, therefore, easier to work with. Photographs of colonies of CN2000 and the other strains considered in this paper are shown in Fig. 1. CN2000D, a strain of CN2000 maintained at the RIJKS Instituut Voor De Volksgezonnheid, Utrecht, The Netherlands, was supplied by R. C. Righelato (31). Weissensee G (originally from the Biogena Institute, Prague) was a substrain received from and reported to

have been "cured" of its *tox*$^+$ character by S. S. Rao, the Haffkine Institute, Bombay, India (29); and the Bikens strain was kindly supplied by T. Hirai (16).

Strains PW8$_r$(Pd) and PW8$_s$ (Pd) of this laboratory (6) are redesignated PW8$_r$(P) and PW8$_s$(P). The results reported herein show them to share the following genetic markers: tryptophan independent (can synthesize indole), yield phage with P·603 phenotype upon induction with ultraviolet light, produce large amounts of diphtherial toxin, and are resistant to bacteriophages which specifically lyse *mitis* strains of *C. diphtheriae*. Under conditions optimal for growth, mean generation times in minutes for the following strains were found: Bikens, 127 min; CN2000, 131 min; CN2000D, 120 min; PW8$_r$(P), 146 min. PW8$_s$(P)$^{tox+}$ grows a little faster (6). The PW8$_s$(P) strain isolated from the SM-1 strain of Yoneda (5) differs from all other PW8 strains in that it requires indole, yields phage with the phenotype P·C7 after induction with ultraviolet light, and is sensitive to certain *mitis*-specific bacteriophages. Miller et al. (26) correctly redesignated this strain P60(β).

Indicator strains. C7$_s$(−)$^{tox-}$, hereafter referred to as C7, is the standard indicator for bacteriophages active on a number of *mitis* strains (6, 7, 18).

Strain 603 was obtained from Sheldon Arden (Goldzimmer, Arden, and Barksdale. Bacteriol. Proc., p. 160, 1968), a strain of *C. ulcerans* originally described by Henriksen and Grelland (15).

Bacteriophages. Strain β is a well-studied phage maintained in this laboratory (18). It was originally derived as a mutant of phage B (7) from Freeman (12) who obtained his stock from Toshach (38). Evidence presented in this paper makes it clear that phages P and β have much in common. The following quotation from a letter from Toshach to one of us (L. B.), dated 8 June 1967, indicates the difficulty in establishing a common origin for phage P and phage β: "I have searched my age-old thesis and my correspondence with Freeman for some clue as to the source of the phage I sent him. His phage B was my C/13. This was obtained from an unholy mixture. The letter C stood for a combination of cultures (three *intermedius*, one *mitis*, and three *gravis* strains plus the supernatant of another *gravis* strain). The supernatant of this (following incubation) revealed plaques on culture 13 (a *mitis* strain) and was subsequently propagated on this culture. This propagating strain I also sent to Freeman. It was apparently my intention to replace the letter C with the number of the lysogenic strain in the mixture, but I note that repeated attempts to propagate the phage on any of the individual strains in the mixture failed; as did attempts to demonstrate phage in supernatants of the individual cultures. Hence the lysogenic strain could have been any of the seven cultures in the mixture or the phage could have been in the supernatant added to the seven cultures initially" (quoted by permission).

Phages P·603 and P·C7, isolated in the course of the present study, are host-modified phenotypes of phage P (4).

Neutralization of phage by specific antiserum. The procedure of Adams (1), for phage neutralization, was followed with an antiserum prepared by Holmes (18).

Carbohydrate supplements. A 40% maltose supplement had the following composition: maltose, 100 g; 1.0% aqueous calcium pantothenate, 0.15 ml; 10% aqueous $CaCl_2 \cdot 2H_2O$, 7.5 ml; and distilled water to 250 ml. A 20% glucose supplement had the following composition: glucose, 50 g; 1.0% aqueous calcium pantothenate, 0.15 ml; 10% aqueous $CaCl_2 \cdot 2H_2O$, 7.5 ml; and distilled water to 250 ml. The supplements were distributed in 50-ml portions in 8-oz (226-g) screw-capped bottles and sterilized by autoclaving at 110 C for 20 min.

Liquid media. For PGT medium, the casein hydrolysate medium of Mueller and Miller (27) was modified to contain added tryptophan, pantothenate, and glutamate as described by Barksdale and Pappenheimer (7), plus 0.001% (w/v) phenol red. The *p*H was adjusted to 6.85 with 50% KOH.

The PGT medium without added tryptophan was used as PG medium. Before using media, sterile 40% (w/v) maltose supplement was added to PG to yield a final concentration of 4% maltose and to PGT to yield a final concentration of 2% maltose.

Solid media. Tryptose-agar medium had the following composition: Tryptose (Difco), 10.0 g; NaCl, 5.0 g; distilled water to 1 liter; and agar [(i) soft agar overlay for phage assay, 5 g; (ii) hard agar for phage assay, 10 g; (iii) hard agar for colony formation, 15 g]. To sterile soft agar (i), one-tenth part of glucose supplement was added aseptically just before use. To hard agar for colony formation (iii), one-tenth part of sterile maltose supplement was combined with molten Tryptose-agar before pouring plates.

Chocolate agar medium was made with sterile neopeptone-agar (500 ml per bottle) of the following composition: Beef Heart Infusion (Difco), 500 g; Neopeptone (Difco), 20 g; NaCl, 5 g; agar, 20 g; distilled water to 1 liter; final *p*H 7.4. The neopeptone-agar was melted, allowed to cool to 80 C, and combined with 35 ml of sterile horse serum and 25 ml of sterile horse blood (previously warmed to room temperature). The bottle was constantly rotated in an 80 C water bath until an even chocolate color was obtained. The mixture was allowed to cool to about 65 C and was then poured into sterile clay-topped petri dishes.

Cystine Trypticase-agar (Difco) was used as a base for fermentation media. The carbohydrates used were glucose, sucrose, maltose, starch, and trehalose in 0.5% (w/v) amounts.

Gelatin medium was made of: Peptone (Difco), 10 g; beef extract, 3 g; NaCl, 5 g; gelatin, 150 g; and distilled water to 1 liter. All ingredients other than the gelatin were dissolved without heating. The *p*H was adjusted to 7.2 to 7.4 with 1 N NaOH. Gelatin was added and dissolved by heating over a boiling-water bath. The mixture was then filtered through cotton and tubed in 7-ml quantities. Sterilization was by autoclaving at 120 C for 15 min.

Phage assay. The procedure of Holmes and Barksdale (18) was followed for phage assay.

Spot tests for detection of bacteriophage. Lawns were made by using 0.1 ml of sensitive indicator bacilli [optical density (OD) of 0.3 for C7 or 0.4 for 603] in 2 ml of Tryptose-soft agar (plus glucose supplement) overlayed on Tryptose-1% agar plates. Onto the dried lawns, so prepared, drops of the phage stocks to be tested were spotted undiluted and at various dilutions.

Preparation of phage stocks. Phage stocks were prepared from single plaques, with two cycles of lysis, by the procedure of Holmes (18).

Measurement of phage adsorption. Log-phase cells of the bacterial strains to be tested (adjusted to OD 0.3) were combined with bacteriophage. The phage-bacterium mixture was shaken for 25 min at 36 C and 240 rev/min. Samples were then centrifuged at high speed, and the phages remaining in the supernatants were measured and compared with the titer of the stock phage.

Preparation of lysogenic bacteria. Lawns of indicator bacteria were prepared as for phage assays. Drops of bacteria-free, filtered phage stocks were placed on these lawns, and the petri dishes containing them were incubated for 2 days at 30 C. An area of confluent lysis was stabbed with a sterile inoculating needle, and the adhering bacteria were suspended in 1 ml of PGT medium. Samples of these suspensions were streaked on Tryptose-agar plates. Sixty single colonies so isolated were picked onto maltose-supplemented Tryptose-agar plates and incubated at 30 C for 48 hr. The plates were then replica plated onto lawns of a sensitive indicator strain. These replica plates were incubated for 24 hr at 30 C and were examined for clear areas. The colony replicas which gave rise to clear areas were cloned and subcultured in liquid medium, and their lysogenic state was proven by demonstrating that (i) there was phage in culture supernatants, (ii) the clones were immune to lysis by homologous phage, and (iii) they yielded increased amounts of phage after irradiation with small doses of ultraviolet light. The lysogenic strains 603(P) and C7(P) were prepared in this manner.

Induction of lysogenic bacteria with ultraviolet light. A log-phase culture was adjusted to OD 0.15, allowed to grow to OD 0.3, placed in a sterile glass dish to a maximum depth of 3 mm, and irradiated for 135 sec [1,750 (±5%) ergs per cm^2 per sec] by using a 15-w General Electric germicidal lamp by the method of Barksdale and Pappenheimer (7). After irradiation, the culture was transferred to a shielded Erlenmeyer flask and shaken in a New Brunswick model G 76 gyratory shaker at 240 rev/min and 36 C. Impending lysis was monitored by following the OD of the culture and by examining, from time to time, Gram-stained smears under a microscope for the presence of elongated bacilli and gram-negative debris. The lysate was usually harvested at about 5.5 hr postirradiation, the cellular debris was removed by centrifugation at 12,000 × *g*, and the supernatant was assayed for its content of bacteriophage.

Tests for toxinogenicity. Intracutaneous tests for diphtherial toxin were done in guinea pigs as described by Barksdale et al. (5). Supernatants of lysates of cells (603 and C7) infected with phage isolated from CN2000D and also the lysogenic cells, 603(P) and C7(P), were tested in this way for their ability to produce diphtherial toxin.

Ramon flocculation test. The samples of toxin listed in Table 1 were assayed by using the flocculation procedure originally described by Ramon (30) modified as follows. Two stock solutions of diphtherial antitoxin from Wyeth Laboratories Inc., Marietta, Pa., were prepared containing 500 and 250 antitoxin units per ml, respectively. These were tested with standard toxin which had been standardized with lot SA-9 standard antitoxin (secondary), kindly supplied by James A. McComb, Biologic Laboratories, Commonwealth of Massachusetts, Jamaica Plain, Mass. The toxin was assayed by using antitoxin in the following amounts (ml): tube 1, 0.01; tube 2 0.02; tube 3, 0.03; tube 4, 0.04; tube 5, 0.05; and tube 6, 0.06. A 1-ml amount of toxin was used in each tube. The antitoxin was diluted in saline to yield either 500, 250, or 100 antitoxin units per ml. The first rough assays were done by using 5 antitoxin units per tube. The final assays were done by using smaller increments. The tubes containing the antitoxin and toxin were placed in a 47 C water bath. Flocculation occurred with 1 hr, and the tube in which flocculation occurred first was taken to indicate the L_f value. For calculating toxin yields, 1 L_f was taken to be equivalent to about 2 μg of toxin protein (21).

Viable counts. Viable counts were made by serially diluting the culture 10-fold in cold PG, and plating by spreading 0.1-ml portions of appropriate dilutions onto Tryptose-1.5% agar plates. Colony counts were made after incubating the plates for at least 3 days at 36 C.

Fermentation tests. The PW8 substrains were grown overnight to an OD of at least 8.0, and 5-ml samples were centrifuged at 12,000 × *g* for 15 min. The supernatants were decanted, and the pellets were suspended in 5 ml of unsupplemented PG. The cultures were centrifuged again, and the pellets were suspended in 2 ml of PG. Carbohydrate media were inoculated with samples of this material, with a separate sterile capillary tube used for each inoculum. The charged capillary tube was pushed to the bottom of the medium and slowly pulled up while releasing the inoculum. The tubes were incubated at 37 C and were observed periodically. The same procedure was followed for the gelatin test.

Density measurements. OD measurements were made by using a Bausch & Lomb Junior spectrophotometer at 590 nm.

RESULTS

Fermentation patterns of the PW8 strains. All five strains fermented glucose and maltose, failed to ferment sucrose, starch, and trehalose, and failed to hydrolyze gelatin. Thus all produce fermentation reactions characteristic of *C. diphtheriae mitis*.

TABLE 1. *Toxin production by five sublines of the PW8 strain*

Strain	Starting inoculum[a]	OD at 54 hr[b]	Toxin protein[c]
CN2000D	1.3×10^8	14.5	4.9
CN2000	1.49×10^8	10.8	3.0
Bikens	1.15×10^8	11.5	3.0
Rao	2.32×10^8	12.0	3.5
$PW8_r$(P)	2.75×10^8	7.5	2.9

[a] Viable units.
[b] Averaged from four flasks for each strain.
[c] Milligrams of toxin protein per 25 ml per flask.

Colony morphology. In Fig. 1 are illustrated the kinds of colonies the various substrains of PW8 form on chocolate agar. Figure 1A shows a rough strain $PW8_r(P)^{tox+}$, heretofore designated as $PW8_r(Pdi)^{tox+}$. Figure 1F shows a colony of $C7_s(-)^{tox-}$, a smooth strain. Other substrains considered in this paper are shown in Fig. 1B–E. Each of them exhibits degrees of smoothness not found in the $PW8_r(P)^{tox+}$ strain.

Pellicle formation and growth in roller tubes. The rougher strains, those whose cells have more hydrophobic surfaces, grew as pellicles when inoculated by flotation onto the surfaces of liquid media. When grown in roller tubes, their cells tended to stick to the glass sides of the tubes. Smooth strains such as $C7_s(-)^{tox-}$ behaved in just the opposite way, i.e., they grew as diffuse suspensions in broth and did not stick to the glass when growing in roller tubes (under nutritionally optimal conditions). The addition of Tween 80 (0.2%, v/v) to liquid media minimized the adherence to glass as well as the tendency of

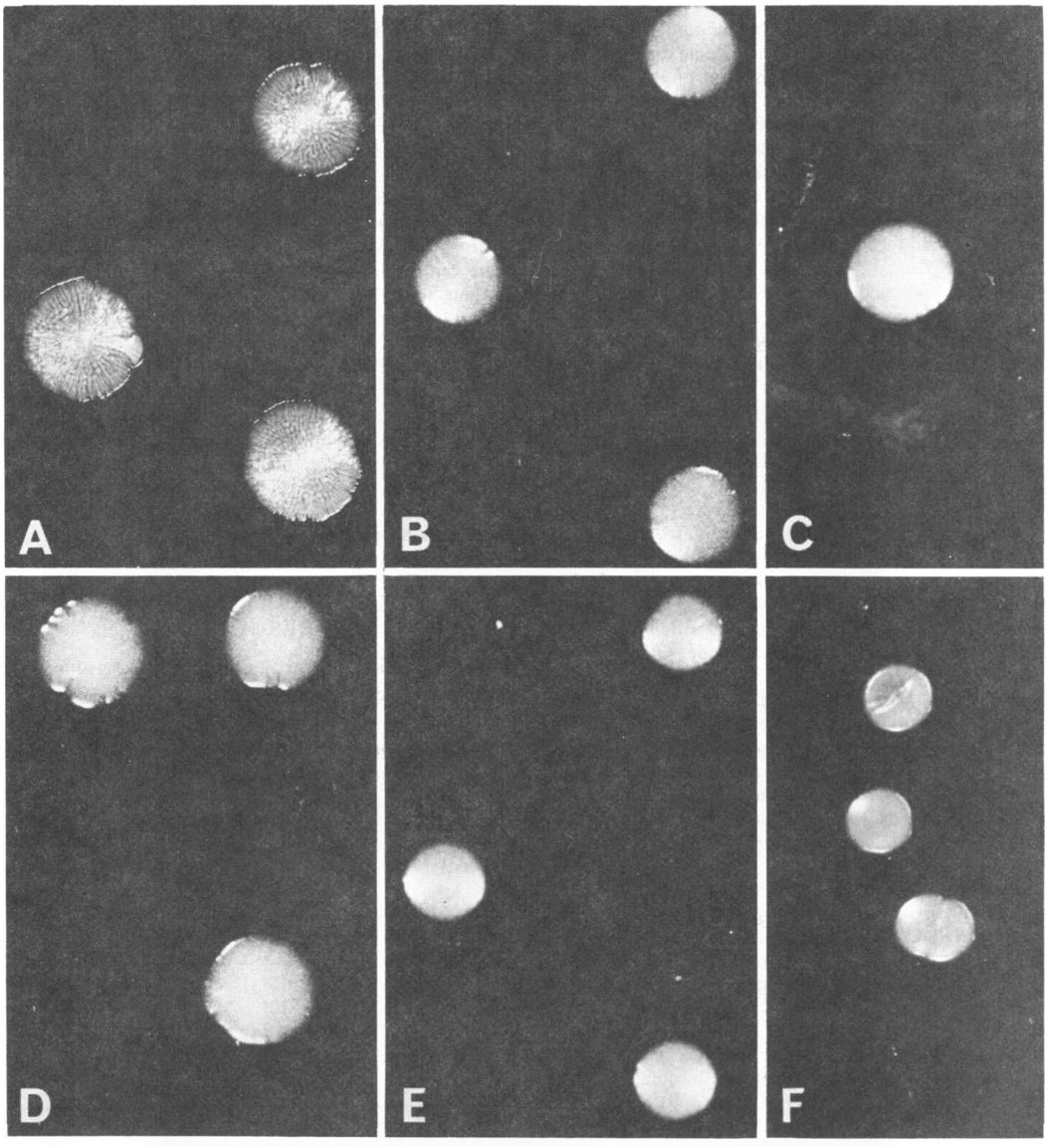

FIG. 1. *Colonies of five substrains of PW8 and the indicator strain grown for 7 days on chocolate agar.* × 3. *A,* $PW8_r(P)^{tox+}$; *B, CN2000; C, CN2000D; D, Rao; E, Bikens; F,* $C7_s(-)^{tox-}$.

rough colonies to form clumps. The presence of Tween 80 did not seem to interfere with toxin production. It has long been known, however, to interfere with the adsorption of corynebacteriophages (13).

Single colonies as inocula for toxin production. The purpose of these experiments was to develop a method whereby the average laboratory could test single colonies (clones) of the PW8 strain for their capacity to serve as inoculum for the production of amounts of diphtherial toxin easily detected by the flocculation test. The *media* employed *were not rendered free of iron* and *acid-cleaned glassware was not used.*

(i) A log-phase culture of each of the strains of PW8 was diluted to yield about 50 colony-forming units per ml, and 0.2-ml amounts of this dilution were plated on chocolate agar plates with a glass spreader. The plates were incubated at 36 C until the average colony diameter was about 0.6 cm (see Fig. 1 and Table 1). By using aseptic precautions and a sterile, stainless-steel spatula (1 cm wide), single colonies were excavated, along with adhering cones of chocolate agar, and transferred to 125-ml Erlenmeyer flasks containing 25 ml of PG medium supplemented with 4% (v/v) maltose. For each strain, four separate colonies were selected.

The average number of cells per colony was determined in the following way. At the time of inoculation, a whole single colony was dug from a chocolate agar plate with a sterile spatula, and the core of agar with the colony was placed in a culture tube containing 10 ml of PG medium and allowed to stand for 5 min. The chocolate agar separated from the colony and was removed with a sterile platinum needle. Two to three glass beads were added to each tube, and the tubes were then agitated on a Vortex mixer for 3 to 5 min. The OD of each suspension was determined at 590 nm. The suspensions were then appropriately diluted and samples were plated to determine the number of viable bacilli present. The results are given in Table 1.

(ii) The flasks were placed in a shaking water bath at 34 C and 240 rev/min and examined from time to time for evidence of an increase in turbidity and for change in color of the phenol red. [It has long been known that in the course of toxin production there is a drop in the *p*H of the medium followed by an "alkaline reversal." Sometimes the rise in *p*H does not occur. We have found detectable toxin in such cases, but the yield can be tripled by neutralizing the medium from time to time with KOH.] OD readings were made on the contents at 20 hr, when an OD of 3 had generally been reached. The shaking speed was then increased to 300 rev/min. To minimize the effect of clumping on OD readings, the samples were agitated with glass beads on a Vortex mixer before reading. The flasks were incubated for a total of 54 hr, at which time the average OD was ± 12.0, or 2.2×10^{10} bacilli per ml, or 0.7 mg of bacterial N per ml (7).

(iii) The cells were removed by high-speed centrifugation, and the supernatants were assayed for toxin by using the flocculation method of Ramon (30) modified as described in Materials and Methods. The range of decrements to be tested was determined by carrying out preliminary flocculation reactions. The amounts of toxin produced by representatives of the various strains are shown in Table 1.

Demonstration of the lysogenic status of the PW8 strains. Cultures (25 ml) of each strain in log phase (OD 0.3) were irradiated with ultraviolet light as described above. The cultures were transferred to aluminum-foil-covered, 125-ml Erlenmeyer flasks and incubated with shaking for 5.5 hr (previously determined as the time to lysis after exposure of the cells to ultraviolet light). Cells and debris were then removed by centrifugation, and the supernatants were assayed for phage content on strain C7. The number of plaques per milliliter of lysate was always small, on the order of 2×10^3 per 10 ml of supernatant when titered the same day as prepared. When the same supernatants were plated on strain 603, the number of plaque-forming units per ml was increased 10^3-fold. Thus two classes of phage could be derived from PW8 lysates: the progeny of those particles which infected strain C7 and behave like phage P already described (4); the other, P·603, the progeny of particles which infect 603. When isogenic stocks were prepared from plaques of P grown on C7, P·C7, and of P grown on 603, each stock was found to plate more efficiently on the host used for its propagation than on the alternate host (*see* Table 3). [The designations P·C7 and P·603 are in keeping with the designations introduced by Arber and Dussoix (3) and Dussoix and Arber (10) for host-modified phenotypes of phage λ; *see also* 2 and 14.] All of the strains of PW8 included in this study could be induced to release phage after irradiation with ultraviolet light, and the phenotype of the released phage was like P·603 and unlike P·C7 (*see* Tables 2 and 3 and Fig. 2).

Number of cells of the PW8 strain induced to lyse by ultraviolet light. To determine the actual number of ultraviolet light-induced cells in which the synthesis of phage particles was taking place, washed PW8 cells (rid of their free phage) were induced by exposure to ultraviolet light and were plated to detect the total number of infective centers. Samples were taken at 55 min after expo-

TABLE 2. *Induction of modifying and nonmodifying P phage lysogens*

Strain (OD 0.3)	Time postirradiation (min)	Free phage (PFU[a]/ml)
$C7_s(P)^{tox+}$	30	0.8×10^6
	150	210×10^6
$C603_s(P)^{tox+}$	35	0.2×10^6
	170	100×10^6

[a] Plaque-forming units.

TABLE 3. *Plaque-forming capacity of P·C7 and P·603*

Phage	Indicator	Bacterium
	$C7_s(-)^{tox-}$	$C603_s(-)^{tox-}$
P·C7	1×10^9	3×10^6
P·603	1×10^1	1×10^{10}

sure to ultraviolet light, long before any of the induced cells would have lysed. The free phage at this time numbered 6,000 particles per ml. The total number of ultraviolet-light-induced infective centers found on lawns of indicator strain 603 was 16×10^6. The unirradiated control contained 210×10^3 infective centers. Since the total number of cells exposed to ultraviolet light was 132×10^6, better than 10% of the irradiated cells yielded a burst of phage capable of lysing strain 603. The number of infective centers found on lawns of C7 was about 100 per 10 ml.

Lysogenization of C. ulcerans 603 and C. diphtheriae C7 with phages P·603 and P·C7, respectively. Drops of phage P·C7 (titer, 10^7 plaque-forming units per ml) and of P·603 (titer, 2×10^9 plaque-forming units per ml) were placed on lawns of C7 and 603, respectively. After 2 days of incubation, resistant bacteria were picked from the lysed areas and streaked out on agar plates. Once C7(P) and 603(P) were obtained as pure lines, cultures of them were tested for the inducibility of their respective prophages by exposure to ultraviolet radiation. That each strain is truly lysogenic (i.e., is not a carrier clone) is seen in Table 2, which shows a rise in free phage after ultraviolet light induction.

Test for expression of tox$^+$ in C. ulcerans strain 603 by P·603 derived from C. diphtheriae PW8. Cultures of strain 603 lysogenized with P·603, and strain C7 lysogenized with P·C7, were separately grown overnight to an OD of ± 8.0 and diluted 100-fold; 0.1 ml of each culture was injected intracutaneously at sites on the depilated back of a 500-g guinea pig. At 4 hr, the animal received 500 units of diphtherial antitoxin. Thirty minutes later identical injections were given at new control sites. At 72 hr, necrotic lesions were formed at the test sites of both 603(P) and C7(P). There was no necrosis at either of the control sites, thus indicating that the toxin produced by each of these strains of bacteria was specifically neutralized by diphtherial antitoxin.

Host-controlled restriction and modification of phage P. When crops of phage P are produced by inducing an endogenous infection in cells of PW8 as a result of exposing them to ultraviolet light, the particles are of the phenotype P·603, i.e., they plate well on 603 and little or not at all on C7. The two phenotypes, P·603 and P·C7, differ not only in their ability to form plaques on the two host strains (*see* Table 3) but also in their stability to storage in cold (*see* Tables 4 and 5). The marked instability of P phage grown in C7 has made for difficulties in experiments with it.

The stable P·603 phenotype was used in the following way to see whether the C7 restriction for it could be lifted. Schell and Glover (34) showed that the capacity of cells of *Escherichia coli* K(P1) to restrict phage can be lowered by starving the cells and then heating them for a

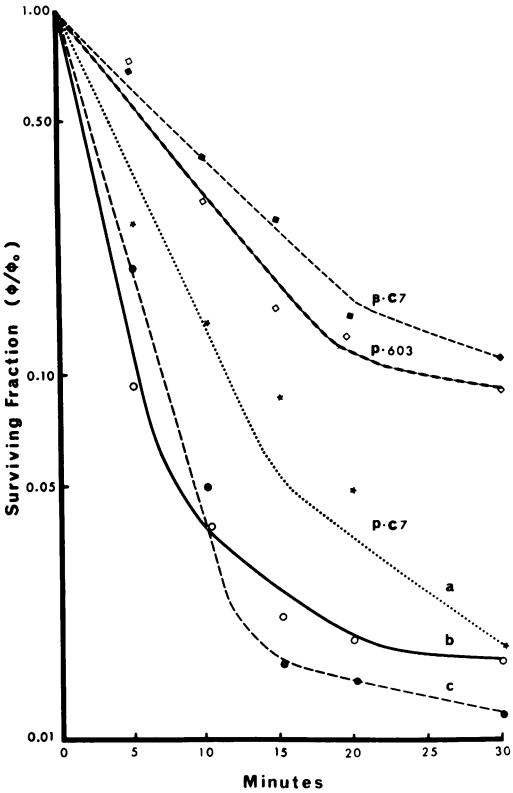

FIG. 2. *Inactivation of β·C7, P·603, and P·C7 by anti-β serum. a, P·C7 derived from strain Bikens; b, P·C7 derived from strain Rao; c, P·derived from P·C7 derived from strain Rao; c, P·C7 derived from strains CN2000D and PW8$_s$(P) yielded curves falling between b and c.*

short time before infecting them. The method of Schell and Glover was applied to the P·603-C7 system as follows. Log-phase cells of C7, OD 0.6, were centrifuged and suspended in 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, *p*H 7.4, containing 0.01 $MgSO_4 \cdot 7H_2O$ and 0.002 M $CaCl_2 \cdot 2H_2O$. The OD was then adjusted to 0.29. Twenty milliliters of this culture was placed in a 250-ml Erlenmeyer flask and shaken for 1 hr to starve the cells. A portion of the C7 (OD had dropped to 0.24) bacilli was heated in glass tubes (inside diameter 14 mm, column height 9 mm) in a 50 C water bath for 10 min. The heated and starved cells, starved but unheated cells, and log-phase C7 were separately combined with P·603, 10^7 plaque-forming units per ml, so that there was a more than 10-fold excess of bacteria to bacteriophage. These three mixtures were allowed to incubate for 25 min. Infected cells were separated from the unadsorbed phage by centrifugation and plated out on lawns of the restricting host, C7. The results, shown in Table 6, show that of the three kinds of C7 cells only those which were both starved and heated show some acceptance (lifting of the restriction) for P·603.

DISCUSSION

Examination of the strains of the diphtheria bacillus of Park and Williams maintained in the United States, England, Holland, Japan, India, and Czechoslovakia has led to some interesting findings. The strains of the bacillus cultivated in widely separated laboratories prove to be remarkably alike. The similarity of their colonial morphologies is illustrated in Fig. 1. They give fermentation patterns characteristic of *mitis* strains. All are lysogenic and all remain good producers of diphtherial toxin (Table 1). After exposure to inducing doses of ultraviolet light, about 10% of the irradiated cells release bacteriophage P. The phenotype of the released phage is such that it cannot plate with satisfactory efficiency on strain C7, but it does plate on *C. ulcerans* strain 603 (Tables 3 and 4). This phenotype is here designated P·603, since, so far as restriction in and modification by C7 is concerned, P phages issuing from PW8 or from 603 are identical. The P·603 phenotype contrasts with P·C7 as follows. (i) The particles are stable to storage in the cold (Table 4), and (ii) they adsorb to but can multiply in a very small fraction of the cells of populations of C7. The restricting capacity of a small number of the cells in populations of C7 can be lifted (inactivated) by subjecting the cells to a combination of starvation and heat (exposure to 50 C; *see* Table 6). In this respect the restriction which operates in C7 behaves like that of *E. coli* strain K-12(P1), which destroys the entering deoxyribonucleic acid (DNA) of phage λ. From the work of Arber and Dussoix (3, 10), Wood (39), Roulland-Dussoix and Boyer (9, 32) and of Meselson and Yuan (25), it seems that the restrictions seen in *E. coli* strains K-12, K-12(P1), and B are imposed by specific endonucleases which act upon unmodified DNA by introducing scissions at a limited number of sites in each of the strands of the DNA duplex. Although these endonucleases, III-K, III-P, and III-B, differ in their substrate (site) specificities, they appear to belong to a single class of endonucleases (25, 32) having about the same molecular weight (300,000) and exhibiting a specific requirement for Mg^{2+}, *S*-adenosyl-L-methionine, and adenosine triphosphate. Although such requirements are absolute in the case of these three nucleases, neither the restriction enzyme(s) which degrades resistance transfer factor-2 nor DNA foreign to strain Rd of *Haemophilus influenzae* has any such requirements (21a, 32, 37). It remains to be discovered which of these models fits the restriction system of C7.

Our most unusual findings with regard to the modified phage P·C7 and its unmodified counterpart, P·603, are (i) the difference in behavior of these two phenotypes in the presence of anti-

TABLE 4. *Stability of P·603 and P·C7 at 4 C*

Assayed on	PFU[a]/ml found on			
	1 day	48 hr	72 hr	10 days
P·603 $C603_s(-)^{tox-}$	1×10^{10}	1×10^{10}	1×10^{10}	2×10^9
P·C7 $C7_s(-)^{tox-}$	1×10^9	3×10^8	1×10^8	3×10^6

[a] Plaque-forming units.

TABLE 5. *Summary of properties associated with phages P·603 and P·C7*

Properties	P·603	P·C7
$C7_s(-)^{tox-}$	Restricted	Permitted
$C603_s(-)^{tox-}$	Permitted	Permitted
To storage at 4 C	Stable	Labile
Inactivation by antiphage β serum	$K = 4.1$	$K = 9.6$

TABLE 6. *Use of starvation and heat to alter the C7 restriction for P·603*

Conditions[a]	PFU/ml found on C7
C7, Log phase	None
C7, starved	None
C7, starved and heated at 50 C for 10 min	2.3×10^3

[a] Cells (10^8 per ml) were exposed to 10^7 plaque-forming units (PFU) of P·603 per ml; 98% adsorbed after 25 min.

phage serum and (ii) the difference in their stabilities to storage in the cold (*see* Fig. 2 and Table 5). L. R. Bullas and R. L. Nutter recently reported similar serological peculiarities and instabilities associated with one of the phenotypes of phage P·3 (from *Salmonella potsdam*) grown on *E. coli* C (Bacteriol. Proc., p. 157, 1969).

Methylation of DNA at specific sites seems to be the modification that enables the DNA of certain phages of *E. coli* to escape restriction (2, 39). An attempt to examine this matter in the case of corynebacterial modifications is being undertaken by using an inhibition-of-modification-and-restriction system similar to that described by Hirsch-Kauffman and Sauerbier (17).

The five PW8 strains whose colonial morphologies are shown in Fig. 1 are somewhat rough. Only $PW8_r(P)$ forms colonies with irregular margins. All five exhibit, however, general attributes of roughness: they grow in broth as a friable pellicle or a sediment and stick to the sides of glass roller tubes and shaking flasks. Of the strains examined, CN2000 and CN2000D were easier to work with because they tended to separate more easily after cell division, especially in Tween-containing media.

The PW8 strains are generally regarded as avirulent organisms. This impression stems from the fact that these bacteria have been handled in batch cultures by numerous laboratory workers over the past six decades with no reports of laboratory infections. In this laboratory one of us (L.B.) has worked with the PW8 strain for 20 years without an infection or even a change in his response to the Schick test (he is Schick-positive, i.e., shows no evidence of circulating antitoxin). In spite of this evidence for the avirulence of the PW8 strain, it was originally isolated from a patient, and occasionally others similar to it have been isolated from patients. It belongs to the serological group K (D-5) of Huang (19). Huang has described cases of diphtheria infected with this serotype and has found that patients so infected respond in a special way to antitoxin therapy. The homologous antibacterial antibodies contained in the antitoxin seem to hasten the disappearance of bacteria from their throats in a manner not seen in persons harboring heterologous strains of *C. diphtheriae*. The toxic dimycolate of trehalose, the cord factor of *C. diphtheriae*, was isolated and characterized from lipids of the PW8 strain (36). Its toxicity was recently studied by Kato (20). The relative avirulence of the PW8 strain suggests that the role of cord factor in virulence is somewhat complex.

The method described here for using entire colonies of diphtheria bacilli as inocula for the production of toxin avoids the tedium involved in preparing cells for toxin synthesis, employs ordinary glassware, and can be carried out in any laboratory equipped with a shaking incubator. The results are extremely reproducible and the amounts of toxin produced are amenable to in vitro methods of assay. Had such a method been available to Rao (29) in 1965, it is unlikely that he would have described his culture of the Weissensee G strain as nontoxinogenic. At that time Rajadhyaksha and Rao reported curing of the Weissensee G strain of the tox^+ gene by treatment with acriflavine, leading them to hypothesize that tox^+ "was carried by a cytoplasmic toxinogenic factor designated as T^+." In our hands the cured strain they sent to us makes 11 μg of toxin per OD unit (*see* Table 1) and is also lysogenic.

Saragea and Maximescu reported (33) that none of the phages in their typing system lyse PW8. Thus, so far as is known, PW8 has no receptors for corynebacteriophages. Since these PW8 strains have no receptors for phage P, should they lose prophage P, there is no way for them to become reinfected. The Park-Williams 8 strain, then, stands as an impressive example of the stability possible between a prophage and its host, for in the innumerable divisions the PW8 genome has gone through since 1896 it has lost neither its prophage, its tox^+ character, nor a number of other properties by which it can be recognized.

ACKNOWLEDGMENTS

We are indebted to Marjorie Christiansen Pollice for independently repeating some of the experiments reported herein and for much help in general.

We are grateful to Kwang Shin Kim for Fig. 1 and to Pao-zun Yeh for Fig. 2. We thank Geraldine Hodgson and Myrna Cort for help with the manuscript.

This investigation was supported by Public Health Service grant AI-01071 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Adams, M. H. 1959. Bacteriophages. Interscience Publishers, Inc. New York.
2. Arber, W. 1965. Host-controlled restriction and modification of bacteriophage, p. 295–314. *In* L. V. Crawford and M. G. P. Stoker (ed.). The molecular biology of viruses. Symp. Soc. Gen. Microbiol. 18th, Cambridge.
3. Arber, W., and D. Dussoix. 1962. Host specificity of DNA produced by *Escherichia coli*. I. Host controlled modification of bacteriophage λ. J. Mol. Biol. **5**:18–36.
4. Barksdale, L. 1955. Sur quelques bacteriophages de *Corynebacterium diphtheriae* et leur hôtes. C. R. H. Acad. Sci. **240**:1831–1833.
5. Barksdale, L., L. Garmise, and K. Horibata. 1960. Virulence, toxigeny and lysogeny in *Corynebacterium diphtheriae*. Ann. N.Y. Acad. Sci. **88**:1093–1108.
6. Barksdale, L., L. Garmise, and R. Rivera. 1961. Toxinogeny in *Corynebacterium diphtheriae*. J. Bacteriol. **81**:527–540.
7. Barksdale, W. L., and A. M. Pappenheimer, Jr. 1954. Phage-host relationships in nontoxigenic and toxigenic diphtheria bacilli. J. Bacteriol. **67**:220–232.

8. Bertani, G., and J. J. Weigle. 1953. Host controlled variation in bacterial viruses. J. Bacteriol. **65**:113-121.
9. Boyer, H. D., and D. Roulland-Dussoix. 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459-472.
10. Dussoix, D., and W. Arber. 1962. Host specificity of DNA produced by *Escherichia coli*. II. Control over acceptance of DNA from infecting phage. J. Mol. Biol. **5**:37-49.
11. Edwards, D. C. 1960. The growth and toxin production of *Corynebacterium diphtheriae* in submerged culture. J. Gen. Microbiol. **22**:698-704.
12. Freeman, V. J. 1951. Studies on the virulence of bacteriophage-infected strains of Corynebacterium diphtheriae. J. Bacteriol. **61**:675-688.
13. Groman, N. B., and K. McCormick. 1961. Relation between adsorption of diphtheria phage and its inactivation by oleic acid-activated inhibitor. J. Bacteriol. **81**:394-400.
14. Hayes, W. 1968. The genetics of bacteria and their viruses, 2nd ed., p. 514-522. John Wiley & Sons, Inc., New York.
15. Henriksen, S. D., and R. Grelland. 1952. Toxigenicity, serological reactions and relationships of the diphtheriae-like corynebacteria. J. Pathol. Bacteriol. **64**:503-511.
16. Hirai, T., T. Uchida, Y. Shinmen, and M. Yoneda. 1966. Toxin production by *Corynebacterium diphtheriae* under growth limiting conditions. Biken J. **9**:19-31.
17. Hirsch-Kauffmann, M., and W. Sauerbier. 1968. Inhibition of modification and restriction for phages λ and T_1 by co-infecting T_5. Mol. Gen. Genet. **102**:89-94.
18. Holmes, R. K., and L. Barksdale. 1969. Genetic analysis of *tox*$^+$ and *tox*$^-$ bacteriophages of *Corynebacterium diphtheriae*. J. Virol. **3**:586-598.
19. Huang, C. H. 1942. Studies on the antibacterial property in diphtheria. Amer. J. Hyg. **35**:317-324.
20. Kato, M. 1970. Action of toxic glycolipid of *Corynebacterium diphtheriae* on mitochondrial structure and function. J. Bacteriol. **101**: 709-716.
21. Katsura, T., I. Kato, H. Nakamura, and J. Koyama. 1957. Purification of diphtheria toxin. Jap. J. Microbiol. **1**:213-234.
21*a*. Kelly, T. J., Jr., and H. O. Smith. 1970. A restriction enzyme from *Hemophilus influenzae*. II. Base sequence of recognition site. J. Mol. Biol. **51**:393-409.
22. Luria, S. W., and M. L. Human. 1952. A nonhereditary, host-induced variation of bacterial viruses. J. Bacteriol. **64**:557-569.
23. Matsuda, M., and L. Barksdale. 1967. System for the investigation of the bacteriophage-directed synthesis of diphtherial toxin. J. Bacteriol. **93**:722-730.
24. Maximescu, P. 1968. New host-strains for the lysogenic *Corynebacterium diphtheriae* Park-Williams No. 8 strain. J. Gen. Microbiol. **53**:125-133.
25. Meselson, M., and R. Yuan. 1969. DNA restriction enzyme from *E. coli*. Nature (London) **217**:1110-1114.
26. Miller, P. A., A. M. Pappenheimer, Jr., and W. F. Doolittle. 1966. Phage-host relationships in certain strains of *Corynebacterium diphtheriae*. Virology **29**:410-425.
27. Mueller, J. H., and P. A. Miller. 1941. Production of diphtheria toxin of high potency (100 Lf) on a reproducible medium. J. Immunol. **40**:21-32.
28. Park, W. H., and A. W. Williams. 1896. The production of diphtheria toxin. J. Exp. Med. **1**:164-185.
29. Rajadhyaksha, A. B., and S. S. Rao. 1965. The role of phage in transduction of the toxinogenic factor in *Corynebacterium diphtheriae*. J. Gen. Microbiol. **40**:421-429.
30. Ramon, G. 1922. Floculation dans un mélange neutre de toxine-antitoxine diphthérique. C. R. Soc. Biol. (Paris) **86**:661-663.
31. Righelato, R. C., and P. A. van Hemert. 1969. Growth and toxin synthesis in batch and chemostat cultures of *Corynebacterium diphtheriae*. J. Gen. Microbiol. **58**:403-410.
32. Roulland-Dussoix, D., and H. W. Boyer. 1969. The *Escherichia coli* B restriction endonuclease. Biochim. Biophys. Acta **195**:219-229.
33. Saragea, A., and P. Maximescu. 1964. Schema provisoire de lysotypie pour *Corynebacterium diphtheriae*. Arch. Roum. Pathol. Exp. Microbiol. **23**:817-838.
34. Schell, J., and S. W. Glover. 1966. The effect of heat on the host controlled restriction of phage λ in *Escherichia coli* K (P1). J. Gen. Microbiol. **45**:61-72.
35. Scholes, P. B., and H. K. King. 1965. Electron transport in a Park-Williams strain of *Corynebacterium diphtheriae*. Biochem. J. **97**: 754-765.
36. Senn, M., T. Ioneda, J. Puddles, and E. Lederer. 1967. Spectrométrie de masse de glycolipides. I. Structure du "cord factor" de *Corynebacterium diphtheriae*. Eur. J. Biochem. **1**:353-356.
37. Smith, H. O., and K. W. Wilcox. 1970. A restriction enzyme from *Hemophilus influenzae*. I. Purification and general properties. J. Mol. Biol. **51**:379-391.
38. Toshach, S. 1950. Bacteriophages for *C. diphtheriae*. Can. J. Pub. Health **41**:332-336.
39. Wood, W. 1966. Host specificity of DNA produced by *Escherichia coli*: bacterial mutations affecting restriction and modification of DNA. J. Mol. Biol. **16**:118-133.