Isolation of a Cured Strain from *Corynebacterium diphtheriae* PW8

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A nonlysogenic, non-toxinogenic strain was isolated from the PW8 strain of *Corynebacterium diphtheriae* by two-step ultraviolet induction. This strain was lysed by phages derived from the PW8 strain but not by β phages from the C7(β) strain. When this cured strain was lysogenized with phages from the PW8 strain, toxin production by the resulting lysogens was about a half or a quarter of that of the parent strain PW8.

Since Park and Williams isolated the PW8 strain of *Corynebacterium diphtheriae* from a patient in 1896 (12), this strain has been used to prepare diphtheria toxin and toxoid because of its high toxoid yields. Good indicator strains, realizing this, was reported previously (4). This paper reports the isolation and some characters of a cured strain resulting from PW8 strain by ultraviolet induction. This cured strain will be useful for studies on toxin hyper-producibility and for phage typing of *C. diphtheriae*.

The nonlysogenic, nontoxinogenic strain C7(-) of *C. diphtheriae* and the 9304 strain of *C. ulcerans* (12) were used as indicator strains. A substrain (Biken) originally derived by single colony isolation from another substrain (Tront Harvard) of *C. diphtheriae* PW8 was used for the isolation of the cured strain. Phages β and ω were prepared from UV-induced C7(β) and PW8.

Deferrated CMS medium (3) or PGT medium (2) was used for toxin production and growth. The antitoxin agar medium for the PW8 strain was the antitoxin agar medium used for the C7(β) strain as reported previously (4) supplemented with yeast extract (5 g/liter). After deferrating the medium (16), agar was added and the mixture was autoclaved at 115°C for 15 min. A final concentration (per milliliter) of 4 or 2 floculating units of horse antitoxin serum (supplied from the vaccine production plant of our institute) was then added, and 20-ml volumes of medium were poured into petri dishes (9-cm diameter).

Cells from exponentially growing cultures of strain PW8 in CMS medium were collected and washed once. They were then suspended at a cell density of approximately 4 × 10^9/ml, and 5-ml portions of the suspension were irradiated in petri dishes (9-cm diameter) under a Toshiba 15-W germicidal lamp (GL-15) at a distance of 45 cm for 30 s with gentle shaking. Then the bacteria were collected, washed with medium, and resuspended in fresh medium. The survivors (ca. 10%) were plated on deferrated tryptose agar plates containing horse antitoxin (4 U/ml), and the plates were incubated at 35°C for 3 days. Then colonies were examined to see whether they had halos of toxin-antitoxin precipitate (4). After the first irradiation, no colonies without halos were found among about 75,000 colonies, but 6 colonies with small halos—without decreased amounts of toxin production—were obtained. These six strains produced about half as much toxin as the parent strain PW8 in liquid medium. One of the six strains was named PW8-23. The growth rates and survival rates after UV irradiation of strains PW8 and PW8-23 were almost the same. PW8-23 was again irradiated with UV for 30 s and plated on deferrated tryptose agar medium containing horse antitoxin (2 U/ml). After the second irradiation, 1 colony without a halo was found among about 20,000 colonies.
and it was named PW8(-)-5. PW8(-)-5 did not release phages that lysed C. ulcerans strain 9304, and it was lysed by phages derived from the PW8 strain. [The growth rates of strains PW8, PW8-23, and PW8(-)-5 were almost the same.] Strains PW8-23 and PW8(-)-5, like strain PW8, did not require tryptophan in the medium for growth. Proteins in the supernatants of cultures of PW8 in CMS medium (Fe\(^{3+}\), 0.1 \(\mu\)g/ml) were precipitated with 70% (NH\(_4\))\(_2\)SO\(_4\) and then examined by sodium dodecyl sulfate–gel electrophoresis (15). Figure 1 shows that no band of toxin at a position corresponding to a molecular weight of about 62,000 was obtained from the supernatant of the PW8(-)-5 strain, but it was obtained from the culture supernatant of the PW8 strain. The culture supernatant of the PW8(-)-5 strain in CMS medium did not form a precipitation line against horse antitoxin on immunodiffusion (11), and it had no detectable toxicity in rabbit skin, whereas the culture supernatant of the PW8 strain in CMS medium was toxic (50 flocculating units per ml, \(5 \times 10^6\) minimum reacting doses per ml).

The plating efficiencies of the phages to various corynebacterial strains are summarized in Table 1. All phages tested did not form plaques on PW8 or PW8-23 lawns. Before using PW8(-)-5 cells for plating, the cell suspension in medium was shaken vigorously with glass beads to obtain a homogeneous suspension. Only the phages released from PW8 and PW8-23 could form plaques on PW8(-)-5 lawns, and they were indistinguishable in plaque morphology and host ranges. The plaques formed by the \(\omega\) phages on PW8(-)-5 lawns were all small and turbid like those on C. ulcerans lawns. The plating efficiencies of UV lysates of the PW8 strain or PW8-23 strain on PW8(-)-5 lawns were about five times greater than those on C. ulcerans lawns. \(\omega\) Phages propagated in C. ulcerans cells (\(\omega_u\)) were restricted, forming plaques on PW8(-)-5 lawns. A very few particles of their phages (\(\omega_u\), \(\omega_u\), \(\omega_u\)) formed plaques on C7(-) lawns. Phages \(\beta\) and \(\omega\) were indistinguishable in host ranges and plating efficiencies that were about 100 times greater on C7 lawns than C. ulcerans lawns. \(\beta\) Phage propagated in C. ulcerans cells (\(\beta_u\)) formed plaques comparatively greater than \(\omega_u\) phages on C7(-) cells. About plating efficiencies, Lampidis et al. determined that of P phage in C. ulcerans 603 and C7 strains (5).

The PW8 strain and its cured PW8(-)-5 strain were compared with adsorption by these phages. PW8 and PW8(-)-5 strains failed to adsorb \(\beta\) or \(\omega\) phages, but adsorbed \(\omega\) and \(\omega_u\) in about 60% to 70% of initial input phages, and \(\omega_u\) and \(\beta_u\) in about 30%. Phage adsorptions to cells were almost the same between PW8 and PW8(-)-5 strains.

When burst size was determined using PW8(-)-5 strain as indicator after UV irradiation of PW8 (Biken strain), it was about 60.

PW8(-)-5 cells were lysogenized with \(\omega\) or \(\omega_u\) at a multiplicity of about 1; newly formed lysogens were obtained by single-colony isolation, and each was established as a homogeneous strain by halo formation. Toxin production by the resulting lysogen were then determined in more than 300 strains. The resulting lysogens produced about half or a quarter as much toxin as the PW8 strain.

It is unknown why toxin production was lower than that of the parent strain PW8. The C7 strain-\(\beta\) phage system has been established, and it has been found that \(\beta\) phage carries the structural gene for diphtheria toxin and many kinds of nontoxic proteins serologically related to the toxin (13, 14). It is certain that the phage of the PW8 strain also carries the toxin structural gene. Since we have isolated a cured strain of PW8, it will now be easier to isolate nontoxic proteins serologically related to the toxin produced by PW8 lysogenized with mutant phage. The toxin yields by C7 strains in PGT medium were about 4 flocculating units per ml, whereas those by C. ulcerans strains were about one-fifth as much (9). Even though toxin production by this lysogen PW8-5 is lower than that of the original PW8, its rate of toxin production is about three

**FIG. 1.** Sodium dodecyl sulfate–gel patterns of proteins from crude culture supernatants of strains PW8 and PW8(-)-5. Cells were cultured in CMS medium containing 0.1 \(\mu\)g of Fe\(^{3+}\) per ml at 35°C for 24 h. The supernatants of the two cultures were concentrated about 40-fold, and 3 \(\mu\)l of each was then subjected to sodium dodecyl sulfate–gel electrophoresis under the conditions described by Weber and Osborn (11) on 10% gels.
Table 1. Plaque-forming efficiencies of ω and β phages

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<thead>
<tr>
<th>Indicator strain</th>
<th>Bacteriophagea</th>
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<tbody>
<tr>
<td></td>
<td>ω</td>
<td>ω-23</td>
<td>ω₃</td>
<td>ω₇</td>
<td>β</td>
</tr>
<tr>
<td>PW8</td>
<td>= b</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>PW8-23</td>
<td>—</td>
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<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PW8(-)-5</td>
<td>5 x 10⁶</td>
<td>1 x 10⁶</td>
<td>1 x 10⁹</td>
<td>5 x 10⁶</td>
<td>6 x 10⁵</td>
</tr>
<tr>
<td>C. ulcerans (-)</td>
<td>1.1 x 10⁹</td>
<td>2 x 10⁶</td>
<td>10⁶</td>
<td>1.1 x 10⁹</td>
<td>3.7 x 10³</td>
</tr>
<tr>
<td>C7(-)</td>
<td>&lt;10</td>
<td>&lt;10</td>
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a ω, Phage released from PW8; ω-23, phage released from PW8-23; ω₃, ω phage propagated in C. ulcerans cells; ω₇, ω purified by several successive single-plaque isolations from a very few particles that formed plaques on C7(-) cells and was propagated in C7(-) cells; β, phage released from C7(β); β₃, β phage was propagated in C. ulcerans cells. Data are shown as number of plaques found in each indicator strain.

b —, No plaque formation.

to five times greater than that by C7(β).

LITERATURE CITED