Association of Bacteriophage Particles with Toxin Production by *Clavibacter toxicus*, the Causal Agent of Annual Ryegrass Toxicity

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ABSTRACT

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The association between *Clavibacter toxicus*, the causal agent of annual ryegrass toxicity in Australia, and a bacteriophage specific to the bacterium was examined. Wild-type *C. toxicus* strains (designated type 1) did not produce corynetoxin, the toxin responsible for annual ryegrass toxicity. When bacteria were infected with the bacteriophage, two types of colonies resulted. One type (type 2) produced toxin and the other (type 3) did not. Antiserum was raised to purified bacteriophage particles. Type 2 bacteria reacted positively to the antiserum in enzyme-linked immunosorbent assay (ELISA) but type 1 and 3 colonies did not. Culture of toxin-producing (type 2) bacteria with antiphage antiserum restored normal colony morphology and eliminated activity in the corynetoxin bioassay. DNA hybridization revealed that bacteriophage DNA was not present in type 1 strains but was present in type 2 and 3 strains. Bacteriophage DNA was not integrated into bacterial DNA and was present in high copy number. Toxin-producing strains had an unusual morphology, and when examined by electron microscopy, the bacterial capsule and cell membrane appeared disrupted. Intact phage particles were visible in transmission electron micrographs of the infected bacteria. Evidence indicated that bacteriophage in toxin-producing bacteria (type 2 colonies) was in a phage-carrier state with *C. toxicus*, and bacteriophage presence was correlated with production of corynetoxin.

Annual ryegrass toxicity is a poisoning of grazing livestock that occurs when a bacterium, *Clavibacter toxicus*, is carried into the developing seeds of ryegrass, *Lolium rigidum* Gaudin, by an *Anguina* nematode, *Anguina funesta* Price, Fisher, & Kerr (15), considered synonymous with *A. agrostis* (Steinbuch) Filipjev by some authors (24). The nematode initiates seed galls, and in some galls, the bacterium predominates. Galls containing the bacterium are highly toxic to grazing animals, but galls containing only the nematode are not toxic. The cause of livestock poisoning is corynetoxin, a tunicamycin derivative that inhibits protein glycosylation (5). The condition is responsible for livestock deaths in Australia (6,26) and South Africa (23).

Little is known about corynetoxin production by *C. toxicus*. The morphology of the bacterium was first described by Bird and Stynes (2) and was initially called *Clavibacter rathayi*. Subsequently, it was shown to be distinct from *C. rathayi* (16,21) and has recently been described as a new species, *C. toxicus* (19). Corynetoxin production by cultured bacteria has never been achieved in vitro despite the existence of a rapid, though non-specific, bioassay (20).

Electron microscopic studies (3,25) demonstrated that toxic ryegrass galls contain particles that resemble bacteriophages. However, there was not enough evidence obtained to establish phage properties for these particles. Eleven years later, Riley and Gooden (17) described a bacteriophage isolated from ryegrass galls that is specific to *C. toxicus* and useful for taxonomic purposes. Recently, the same bacteriophage was isolated from *C. toxicus* galls on two new plant hosts, *Agrostis avenacea* C.C. Gmelin and *Polypogon monspeliensis* (L.) Desf., in which corynetoxin is produced (11).

This paper describes the correlation between bacteriophage presence and corynetoxin production and presents preliminary evidence about the nature of the association between the bacteriophages and *C. toxicus* strains.

MATERIALS AND METHODS

Bacterial strains and bacteriophage isolates. *C. toxicus* strains CS2 and CS14 (NCPPB 3552) were isolated from *L. rigidum* from South Australia and strain CS28 (ICMP 9525) was isolated from *L. rigidum* from Western Australia. Bacterial strains were grown and maintained on 523 M agar (19) at 26 C, unless otherwise indicated. The bacteriophage isolate NCPPB 3778 was originally isolated from toxic ryegrass galls (17). Other bacteriophages (R1, N-1, and S-1) were isolated from toxic ryegrass, *A. avenacea*, and *P. monspeliensis*, respectively. All bacteriophage preparations were stored at 4 C in SM buffer (22) amended with 0.5% chloroform.

Sensitivity to bacteriophage. Lawns of bacterial strains to be tested were made using soft (0.5%) agar overlays seeded with indicator strains at 10^8 cells/ml. Aliquots of bacteriophage were added to the overlays, and plates were examined for plaques after 24–48 h of incubation.

Isolation of toxin-producing bacteria and corynetoxin bioassay. Uninfected *C. toxicus* strains were designated type 1. Broth cultures (100 ml) of type 1 strains CS2, CS14, and CS28 were grown for 2 days at 25 C (10^8 cells/ml) when approximately 10^8 plaque-forming units (pfu) of the four bacteriophage isolates were added. Cultures were incubated for 10–14 days; lysis occurred after 2–3 days of incubation at 25 C. Aliquots (200 μl) of lysate (10^9 pfu/ml) were plated on 523 M agar, and two colony types appeared after incubation. Colonies that grew 5–7 days after the lysate was plated and that had a normal morphology were designated type 3, whereas colonies that grew slowly and were glassy in appearance were designated type 2. Both colony types appeared after lysis of all *C. toxicus* cultures tested. The relative frequencies of their occurrence varied with the strain infected and ranged from one to 10 in 10^8 cells infected for type 2 and from 15 to 50 in 10^8 cells for type 3. Colonies of all three types were tested for corynetoxin production. The bioassay described by Riley and Ophel (20) was used. Putative toxin-producing strains were grown for 2 wk on 523 M agar and then were overlaid with 0.5% buffered

agar containing 10^6 cells/ml of *C. tritici* strain CS4. Agar material taken from zones of clearing were extracted, purified by reverse phase chromatography, and assessed for transferase inhibition typical of purified corynetoxin. All results were consistent with the presence of corynetoxin within the zones of clearing associated with type 2 bacteria (S. Stuart, *personal communication*).

Purification of bacteriophage and bacteriophage DNA isolation. Lysates (1 L) of CS14 were produced, as described above. Bacteriophage particles were purified and their nucleic acid extracted according to the method of Sambrook et al (22). The presence of DNA or RNA was detected by digestion with RNase or DNase respectively. Phage genome size was determined by digestion with restriction enzymes (Boehringer Mannheim, Castle Hill, Australia) under the conditions recommended by the manufacturer, and lambda DNA was digested with *Hin*dIII and *Eco*RI, as a size marker.

Production of antiphage antiserum. Approximately 10^{10} pfu of purified phage preparation was diluted 1:5 in phosphate buffered saline, pH 7.4 (PBS; contains per liter: 8 g of NaCl, 0.2 g of KH_2PO_4, 2.9 g of $Na_2HPO_4 \cdot 12H_2O$), and mixed 1:1 with Freund's complete adjuvant (Calbiochem, San Diego, CA) before subcutaneous injection into a rabbit. Subsequent injections were made subcutaneously every 2 wk. Blood was collected at 5 and 7 wk after primary immunization. Blood was permitted to clot for 1 h at 28 C; serum was decanted, centrifuged (1,500 *g* for 10 min), and stored at −20 C. Titer of antisera was tested initially by Ouchterlony gel diffusion, as described by Riley (16), against a purified bacteriophage preparation.

Passage of bacteria through antiphage antiserum. Type 1 (CS14 and CS28), 2 (CN-3 and CN-5) and 3 strains (CN-8 and CN-18) were streaked on both 523 M agar and 523 M agar amended with filter-sterilized (0.45 μm; Millipore Corporation, Bedford, MA) antibacteriophage antiserum at 5 μl/ml. Plates were incubated at 26 C, and colony morphology was observed after 2 wk. Several colonies were picked from each plate and tested by the corynetoxin bioassay of Riley and Ophel (20) and for reaction in an ELISA (enzyme-linked immunosorbent assay) system.

ELISA. Immunoglobulin G (IgG) was purified from whole antiphage antiserum. An aliquot (500 μl) of antiserum was diluted 1:10 in SDW (sterile distilled water). An equal volume of saturated $(NH_4)_2SO_4$ was added, and the mixture was incubated for 1 h at 4 C before centrifugation (3,000 *g* for 10 min). The precipitate was dissolved in half-strength PBS, was dialyzed overnight against three changes of half-strength PBS, and was passed through a DE-22 column (Whatman, Maidstone, England; 1 g/10 ml) that had been washed and equilibrated with half-strength PBS. Fractions (1 ml) were collected, and the absorbance (A_{280}) was measured. Three fractions with an absorbance greater than 2.0 were pooled, and the IgG concentration was adjusted to 1 mg/ml and stored at −20 C.

Purified IgG was conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) at a rate of 2.5 mg of alkaline phosphatase per milligram of IgG. The mixture was sedimented at 8,000 *g* for 10 min at 4 C, and purified IgG (1 mg/ml) was added directly to the pellet. After dialysis against three changes of half-strength PBS, glutaraldehyde was added (final concentration 0.06%), and the mixture was incubated for 6 h at 26 C. The mixture was dialyzed against three changes of half-strength PBS amended with sodium azide (0.2 g/L) before the addition of 5 mg/ml of bovine serum albumin and was stored at 4 C.

Log-phase bacteria were harvested and diluted in PBS containing 0.05% Tween 20 (PBST) to a concentration of 10^8 cells/ml for use in ELISA. Healthy ryegrass seeds, cv. Wimmera, nontoxic *Anguina* nematode-infected galls, and toxic bacteria-infected galls were collected from two sites. Toxic material from seedheads of *A. avenacea* was collected from two sites. For ELISA tests on ryegrass plant material, 10 seeds or galls were ground in a mortar and pestle in 1 ml of PBST. For infected *A. avenacea*, 30 mg of crystalline material from severely galled seedheads was ground in 1 ml of PBST. Microtiter plates were coated with IgG, diluted 1:500 in carbonate buffer (0.5 M sodium carbonate, pH 9.6; 100 μl/well) and incubated at 4 C overnight. Plates were washed three times with PBST. Antigens were diluted in PBST, 100 μl/well added, incubated overnight, and washed, as described above. Antiphage IgG conjugated to alkaline phosphatase (1:1,000, diluted in PBST) was added (100 μl/well), and plates were incubated at 26 C for 4 h and then washed. Alkaline phosphatase substrate (Sigma Chemical Co.) was added (100 μl/well) and incubated at room temperature. Absorbance (A_{405nm}) was measured after 30- and 60-min incubation with a Bio-Rad model 2550 EIA reader (Bio-Rad Laboratories, North Ryde, Australia).

DNA hybridizations. Total DNA was isolated from three type 1 strains (CS2, CS14, and CS28), two type 2 strains (CR-3 and CN-3), and two type 3 strains (CN-8 and CN-18) by the method described by Hunter (7). Bacteriophage DNA was isolated, as described, digested with *Eco*RI, and labeled with digoxigenin-11-dUTP according to the protocol recommended by the manufacturer (Boehringer Mannheim). Bacterial DNA was digested with *Eco*RI, electrophoresed, and Southern blotted onto Hybond N+ nylon membrane under the conditions recommended by the manufacturer (Amersham, North Ryde, Australia). Hybridization was performed at 42 C overnight in hybridization buffer containing 50% formamide, 5× SSC (1× SSC is 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0), 2% skim milk powder, 0.1% N-lauryl sarcosine and 0.2% SDS (sodium dodecyl sulfate). The membrane was washed under high-stringency conditions (2× SSC, 0.1% SDS at room temperature, followed by 0.1× SSC, 0.1% SDS at 68 C). Digoxigenin detection with the chemiluminescent substrate (Lumigen PPD: 3-(4-methoxyspirol-(1,2-dioxetane-3,2′-tricyclo-decan)-4-yl) phenylphosphate) was performed as recommended by Boehringer Mannheim.

Microscopy. For light microscopy studies, bacteria from late log-phase cultures were diluted in SDW and examined with an Olympus Vanox AHBT microscope (Olympus Optical Co. Ltd., Tokyo, Japan) fitted with differential interference contrast optics.

For scanning electron microscopy (SEM), bacteria in SDW were added as a drop to the surface of a polished carbon stub, allowed to dry at 23 C, and gold-coated prior to examination with a Cambridge S250 MK3 SEM (Cambridge Instruments, Cambridge, England) operated at 20 kV.

For transmission electron microscopy (TEM), bacteria were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.3) for 3 h at 0 C and for 18 h in the same fixative at 23 C. Bacteria were washed by centrifugation in sodium phosphate buffer, washed twice in SDW, stained in 1% aqueous osmium tetroxide for 1 h at 23 C, and washed three times in SDW. Bacteria were dehydrated in an ethanol series consisting of 10, 20, 30, 50, 70, 80, 90%, and absolute ethanol, ethanol/propylene oxide (1:1) through into pure propylene oxide and was left overnight

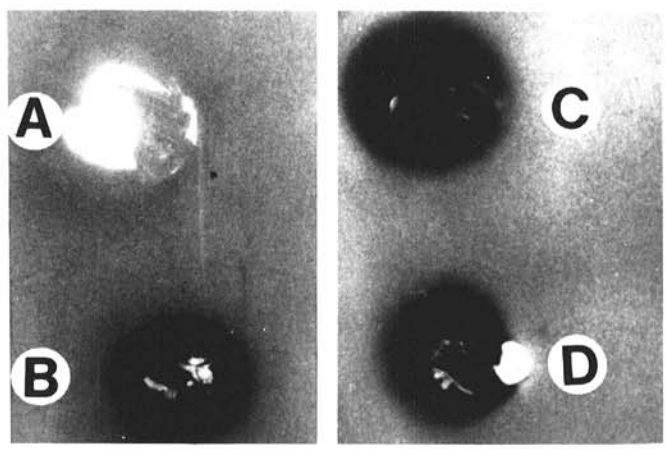

Fig. 1. Corynetoxin bioassay. Zones of inhibition of indicator strain *Clavibacter tritici* CS4 produced by *C. toxicus* strains: **A,** type 3 strain CN-8; **B,** type 2 strain CR 2.1; **C,** type 2 strain CR-3; and **D,** type 2 strain CF 5.6 (mixed with a revertant to a type 3 colony). All bacterial strains were grown in 523 M agar for 2 wk before being overlaid with the indicator.

once in vacuo at 23 C, in a 1:1 mixture of propylene oxide/TK3 resin (TAAB Laboratories Equipment Ltd., Aldermaston, England), and a second time in pure TK3 resin. Bacterial clumps were placed in molds in fresh resin and polymerized at 60 C for 16 h. Sections were cut with a diamond knife in a Reichert-Jung Ultracut E-ultramicrotome (Reichert-Jung Optische Werke A. G., Vienna, Austria). These sections were stained in freshly made aqueous 2% uranyl acetate for 30 min at 23 C, washed six times in SDW (filtered through a 0.2-μm membrane), stained in freshly made 0.5% lead citrate in a 0.1 M solution of sodium hydroxide for 10 min at 23 C, and washed once in 0.1 M sodium hydroxide and six times in filtered SDW. Sections were examined with a Philips EM 400 TEM (Philips, Eindhoven, Netherlands) at 80 kV.

An aliquot of purified bacteriophage preparation was dialyzed twice against a 1,000-fold volume of 100 mM NaCl; 50 mM Tris-HCl, pH 8.0; 10 mM $MgCl_2$ at 4 C. Dialyzed preparation (diluted 1:10 in SDW) was added to a carbon-coated grid, stained with aqueous 2% uranyl acetate for 2 min, blotted, allowed to dry, and examined under TEM (JEM 100CX with scanning attachment; JEOL, Tokyo, Japan).

RESULTS

After infection with bacteriophage in broth culture, wild-type *C. toxicus* strains (type 1) lysed after 2–3 days. When the lysate was incubated further and plated, two types of colonies grew—one with a normal morphology (designated type 3), which appeared 5–7 days after plates were incubated, and one with a glassy appearance (designated type 2), which grew after 14–21 days. The latter colonies were extremely sticky and had a consistency resembling melted cheese. Type 1, 2, and 3 isolates were tested for toxin production and for sensitivity to infection by three bacteriophage isolates. Type 2 isolates produced zones of inhibition in the corynetoxin bioassay (Fig. 1) but type 1 and 3 colonies did not. The presence of corynetoxin in bioassay plates of type 2 strains was confirmed by a transferase assay (S. Stuart, *personal communication*). Type 1 strains were lysed by all four bacteriophage isolates tested but type 2 and 3 colonies were not (Table 1). When subcultured, type 2 colonies grew slowly and sometimes reverted to colonies of normal morphology. When these revertant colonies were examined, they were type 3, based on the criteria shown in Table 1.

TABLE 1. Origin and characteristics of *Clavibacter toxicus* colony types

Strain type	Origin[a]	Corynetoxin activity[b]	Bacteriophage sensitivity[c]	Reaction to antiphage antibody[d]
Type 1:				
CS2	*L. rigidum*, SA	no	yes	no
CS14	*L. rigidum*, SA	no	yes	no
CS28	*L. rigidum*, WA	no	yes	no
Type 2:				
CR-2.1	CS2 with R-1	yes	no	yes
CR-3	CS14 with R-1	yes	no	yes
CR-5.6	CS14 with R-1	yes	no	yes
CN-3	CS14 with N-1	yes	no	yes
CN-5	CS28 with N-1	yes	no	yes
Type 3:				
CN-8	CS14 with N-1	no	no	no
CN-18	CS14 with N-1	no	no	no
CR 4.5	CS14 with R-1	no	no	no
CR 4.6	CS14 with R-1	no	no	no

[a]Type 1 strains were isolated directly from toxic galls (SA = South Australia, WA = Western Australia). Type 2 and 3 strains arose after infection of type 1 strains by bacteriophage isolates R-1 or N-1.
[b]Tested in corynetoxin bioassay of Riley and Ophel (20).
[c]Tested for sensitivity to bacteriophages NCPPB 3778, R-1, N-1, and S-1.
[d]Reaction in enzyme-linked immunosorbent assay to antibody raised to purified bacteriophage NCPPB 3778. $A_{405} > 0.2$ after 30 min was considered a positive reaction.

The morphology of type 1 and 3 strains did not differ after growth on 523 M agar with or without added antiphage antiserum. Type 2 strains produced small, sticky colonies on 523 M agar alone but had a normal morphology on 523 M agar amended with antiserum. Only type 2 strains cultured on 523 M agar alone were active in the toxin bioassay, producing zones of inhibition 19–32 mm in diameter. All other isolates, including type 2 isolates cultured on medium plus antiserum were negative for toxin production.

In ELISA, all type 2 strains reacted positively to antibacteriophage IgG ($A_{405} = 0.268$–0.512) as did the purified bacteriophage particles ($A_{405} = 0.942$ for 10^8 pfu of bacteriophage). Type 1 strains CS2, CS14, and CS28 did not react to antiphage antibody nor did type 3 colonies ($A_{405} < 0.1$) (Table 1). Toxic bacterial galls from ryegrass and *A. avenacea* reacted positively ($A_{405} = 0.230$–1.195) to purified antiphage IgG, but the nontoxic nematode galls and healthy ryegrass seed did not react ($A_{405} < 0.1$).

DNA hybridizations of labeled bacteriophage DNA to bacterial DNA revealed that bacteriophage DNA was present in type 2 and 3 strains but not in type 1 strains (Fig. 2). The pattern of hybridization of bacteriophage DNA to DNA from type 2 and 3 strains suggested that the bacteriophage DNA was not integrated into chromosomal bacterial DNA. The pattern of bacteriophage DNA fragments after restriction was the same for DNA from purified bacteriophage and for DNA from type 2 and 3 strains. The strength of the hybridization signal suggests that a large number of copies of the bacteriophage genome are present in each bacterial cell. There was very weak homology observed between type 1 strains and bacteriophage DNA (Fig. 2), but these bands did not correspond to any of the bacteriophage fragments. When labeled bacterial DNA from type 1 strain CS14 was hybridized

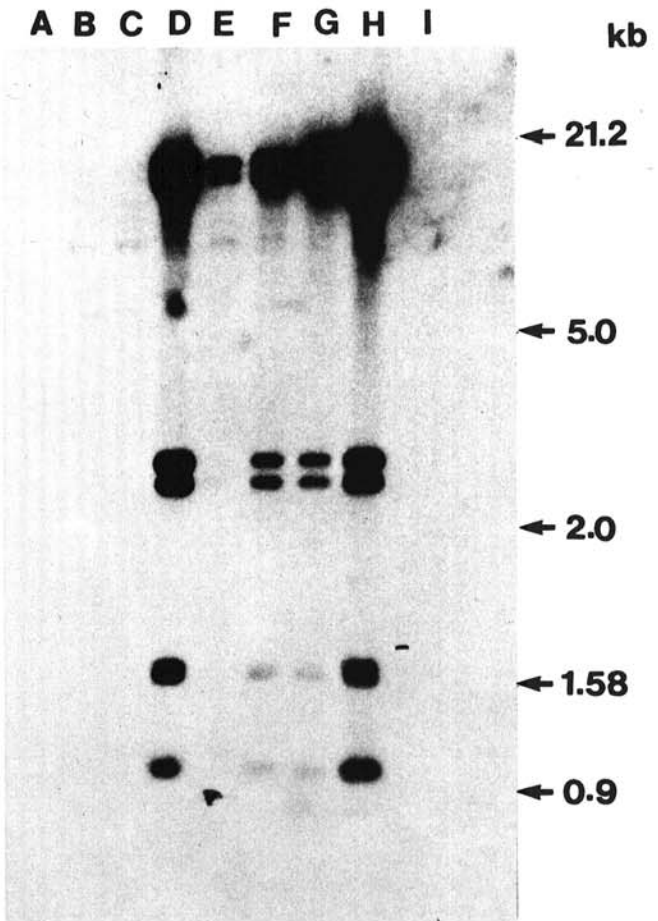

Fig. 2. Southern blot of digoxigenin-11-dUTP-labeled bacteriophage NCPPB 3778 DNA hybridized to *Eco*RI-digested DNA from lanes A, CS2; B, CS14; C, CS28 (type 1 strains); D, CN-8; E, CN-18 (type 3 strains); F, CR-3; G, CN-3 (type 2 strains); H, bacteriophage NCPPB 3778; and I, lambda DNA. Size markers are in kilobases.

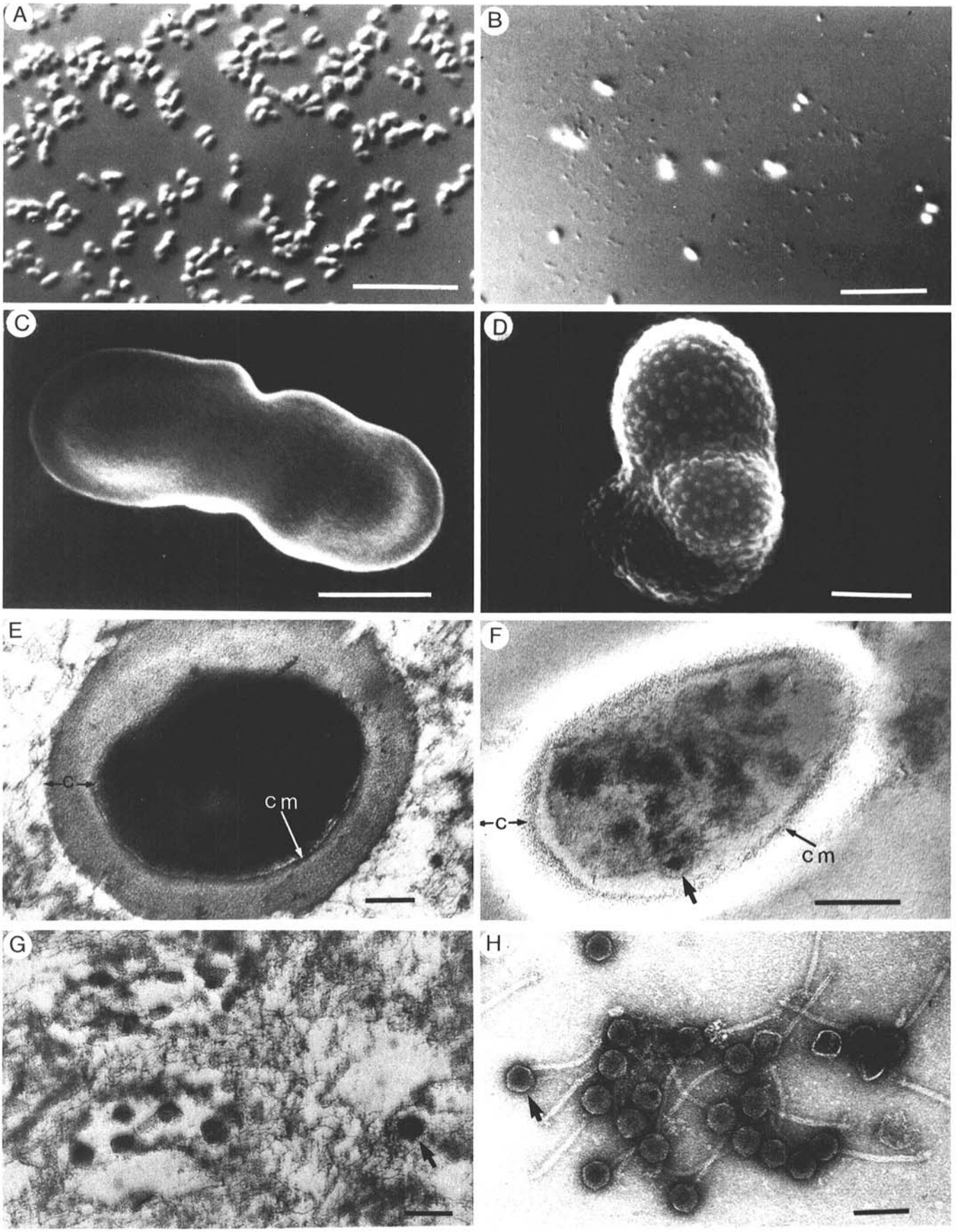

Fig. 3. Differential interference contrast photomicrograph showing **A,** type 1 *Clavibacter toxicus* cells (CS14) and **B,** type 2 strain CN-3. Scale = 10 μm. Scanning electron micrograph of **C,** CS14 and **D,** CN-3. Scale = 0.5 μm. Transmission electron micrograph of a section cut through **E,** strain CS14 and **F,** strain CN-3. Scale = 0.1 μm. Arrow indicates possible bacteriophage head. Deterioration of the capsule (c) and cell membrane (cm) is evident in strain CN-3. **G,** transmission electron micrograph of a section cut through an area showing internal disruption of strain CN-3. Arrow indicates possible bacteriophage head. Scale = 0.1 μm. **H,** transmission electron micrograph of purified bacteriophage particles, stained with 2% uranyl acetate. Arrow indicates bacteriophage head. Scale = 0.1 μm.

to bacteriophage DNA no homology was observed (results not shown). This suggests that the very weak homology observed when bacteriophage DNA was hybridized to type 1 strains was probably due to remnant bacterial DNA coisolated with the bacteriophage preparation.

The morphology of phage-infected and uninfected *C. toxicus* differed strikingly. Under light microscopy, the type 1 *C. toxicus* cells appeared smooth (Fig. 3A), whereas the type 2 cells (Fig. 3B) did not. Under SEM, the capsule of type 2 cells (Fig. 3D) had a rough appearance, whereas the capsule of the wild-type cells (Fig. 3C) appeared smooth. These differences were also observed in TEM sections of type 1 (Fig. 3E) and 2 (Figs. 3F) cells. The dark cytoplasm typical of *C. toxicus* (2) is not evident in type 2 cells. Both the cell membrane and capsule appeared to be disrupted in type 2 cells. In type 2 cells, particles approximately 40 nm in diameter that resembled bacteriophage heads were visible inside most cells (Fig. 3G). No bacteriophage tails were visible. Type 3 colonies were not examined by electron microscopy.

When intact bacteriophages were examined under TEM (Fig 3H), phage-head particles were approximately 35–40 nm in diameter with flexible tails 100- to 130-nm long. The ratio of tail-length to head-length was 2.4 to 2.78, and tail-length to head-width was 2.2 to 2.9. Phage nucleic acid was double-stranded DNA and was approximately 45 kb. Phage DNA was restricted by *Ava*I, *Bam*HI, *Cla*I, *Eco*RI, *Hind*III, *Hpa*I, *Pst*I, and *Sal*I but not by *Xho*I.

DISCUSSION

A positive correlation was found between bacteriophage presence in *C. toxicus* and corynetoxin production. Only *C. toxicus* infected with bacteriophage and producing bacteriophage structural proteins (designated type 2 colonies) produced toxin. This was true for all *C. toxicus* strains and bacteriophage isolates tested.

Bacteriophage DNA was detected only in type 2 and 3 strains and explains why they are resistant to bacteriophage infection. The DNA was not integrated into bacterial chromosomal DNA. The strength of the hybridization signal implies there are a large number of bacteriophage particles in each cell. If the bacteriophage was in a lysogenic association with the bacterium, one would expect that the bacteriophage DNA would be integrated into the bacterial chromosome, and there would be only one copy of the bacteriophage genome per cell. Also, new restriction fragments would be produced. Bacteriophage proteins were present only in type 2 colonies. In a lysogenic association, the only protein produced by the bacteriophage in this state would be a repressor protein. Bacteriophage structural proteins would not be present. It appears that toxin-producing (type 2) cells are not in a lysogenic state but are in a phage-carrier or pseudolysogenic state. A phage-carrier state is an association between a lytic bacteriophage and a susceptible bacterium, and the ways in which this can arise are described by Barksdale and Arden (1). They further define the phage-carrier state as distinguishable from true lysogeny by the passage of phage-infected bacteria through antiphage antiserum (1). True lysogens will be unaffected, but phage carriers will revert back to the wild-type state. The reversion to normal morphology and lack of toxin production after passage through antiphage antiserum in this study confirm that the toxin-producing (type 2) bacteria are phage carriers and not true lysogens. Type 3 strains do not produce bacteriophage proteins but carry multiple copies of an intact bacteriophage genome. The bacteriophage may be present as a plasmid in these strains, as has been described for other bacterial/bacteriophage associations (1), although the mechanism by which this arises is not understood.

Toxin production by phage carriers has been noted for group A streptococci (10,12) and by lysogenic bacteria for *Corynebacterium diphtheriae* (13). Profound biochemical and morphological changes have been described for phage-carrier states in *Shigella dysenteriae* (9), *Brucella abortus* (8), and *Proteus mirabilis* (4). An association of this type has not been reported previously for a plant pathogen.

The presence of bacteriophagelike particles in toxic ryegrass was noted by Bird et al (3). They observed hexagonal particles 25–30 nm in diameter in the capsule of bacterial cells in toxic galls but not in cultured bacteria. The appearance of the bacteriophagelike particles in galls is consistent with that of the particles seen in this study in cultured, toxin-producing cells. These particles were not observed in wild-type, nontoxic cells. The bacteriophage-like particles observed by Bird et al (3) were clustered in the capsule and were smaller than the bacteriophage observed in this study. The discrepancy in particle size may be an artifact of the different methods of processing in sectioning techniques. This explanation seems probable because the appearance and sizes of the structures observed in this study under the SEM were very similar to freeze-fractured material observed previously under the TEM by Bird et al (3). In toxin-producing cells, phage particles were observed inside the cell itself; in toxic galls, they were also observed in the capsule and in the gall walls (3). The toxin-producing cells were not stable in culture, and there may be conditions in planta that stabilize the phage-carrier state and influence particle distribution.

The mechanism by which the bacteriophage affects toxin production is unknown. The structural genes for diphtheria-toxin production are encoded by the bacteriophage (13). This is probably not the case for *C. toxicus* because of the complex nature of the glycolipid corynetoxin molecule. It seems unlikely that the small bacteriophage genome could encode the genes for the toxin as well as those necessary for bacteriophage replication and survival. The bacteriophage may encode a regulatory gene that switches on toxin production or a gene encoding a final step in the corynetoxin biosynthetic pathway. Alternatively, it is possible that bacteriophage infection makes cells more permeable to export of toxin. The bacterial cell membrane appears diffuse in bacteriophage-infected cells and appears to be disintegrating, possibly making the cells more permeable to toxin export.

Toxic bacterial galls from ryegrass reacted positively to antiphage antiserum, indicating the presence of bacteriophage proteins in toxic galls, and the same bacteriophage can be isolated from a range of toxic galls (11). However, toxin-producing colonies are not found in routine isolations from toxic galls, and the bacteria that are isolated are nontoxic type 1 colonies. It seems likely that the bacterium may exist in several states in ryegrass galls, but type 1 colonies are most easily isolated because of their faster and more stable growth. In in vitro studies, type 2 colonies are very difficult to purify because of their growth habit, and these strains are often a mixture of types 2 and 3. Further work will examine the dynamics of bacterial and bacteriophage populations in developing ryegrass galls.

The selective advantage for the association between *C. toxicus* and its bacteriophage is not obvious. Pathogenicity of cultured *C. toxicus* on ryegrass has never been demonstrated, although it has been demonstrated with field-collected bacterial galls (14,18). It is tempting to speculate that the bacteriophage may play a role in pathogenicity or survival of *C. toxicus*. Production of toxin by these infected bacteria may be a reaction to infection, or, alternatively, it may give these slow-growing bacteria a competitive advantage in ryegrass galls.

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