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Detection and Expression of DNA Homologous to the *tox* Gene in Nontoxinogenic Isolates of *Corynebacterium diphtheriae*

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Three probes have been described which can be used to detect the presence of DNA sequences homologous to the *tox* gene of *Corynebacterium diphtheriae*. Probes "A" and "B" detected sequences coding for A and B fragments of diphtheria toxin, respectively. The third "A-B" probe contained both the A and B coding sequences. The B probe was completely unambiguous in detecting only toxin-related sequences, and the A probe was only slightly less so. The efficacy of the probes was tested on a series of toxinogenic and nontoxinogenic isolates of *C. diphtheriae*. All isolates which were toxinogenic as characterized by the gel immunodiffusion technique gave positive reactions with the probes. Of particular interest was the finding that 14 of 43 nontoxinogenic isolates also carried DNA homologous to both the A and B probes. All 14 isolates were nontoxinogenic by the rabbit intracutaneous test as well as by the gel immunodiffusion test; however, 12 of them produced ADP-ribosylating activity, whereas two were negative. The isolates producing ADP-ribosylating activity belonged to a cohort of cultures, of which 11 were isolated in South Dakota and 1 was isolated in Montana. Genomic DNAs of all 12 appeared to be identical when restriction enzyme digest patterns were compared, and the same fragment carried the *tox* gene in all of them. The *tox*-bearing nontoxinogenic isolates from Alaska and Florida each had unique restriction patterns and did not produce ADP-ribosylating activity. A number of genomic fragments of all the *tox*-bearing nontoxinogenic isolates hybridized with β converting phage DNA. The significance of these observations to the natural history of diphtheria was discussed.

The role of non-toxin-producing strains of *Corynebacterium diphtheriae* in the natural history of diphtheria is an intriguing question. The discovery of Freeman (6) that Tox^- *C. diphtheriae* could be converted to the Tox^+ state by beta corynebacteriophage dramatically demonstrated that nontoxinogenic strains could be recruited to toxinogeny. Parsons revealed an additional dimension when she showed that under certain conditions Tox^+ strains could emerge after exposure of a Tox^- strain to the lysate of a second Tox^- strain (16). Parsons' observations were confirmed in this laboratory (7), and the data indicated that in this instance as well, converting phage was responsible for the emergence of the Tox^+ strains. These latter studies suggested that Tox^- strains might themselves carry all or part of the *tox* gene, and that under certain conditions a fully expressed *tox* gene could be recovered.

Whether nontoxinogenic strains of *C. diphtheriae* carry the *tox* gene in whole or in part is in itself a significant question for the epidemiology of diphtheria. Some time ago we reported the isolation of gamma nonconverting phage from a nontoxinogenic strain of *C. diphtheriae* (8), and we subsequently presented both genetic and physical evidence demonstrating the presence of all or part of the *tox* gene in this phage (2, 12). From our data we concluded that nonexpression of the *tox* gene was due to the insertion of a piece of bacterial DNA either in a regulatory site or early in the structural gene for diphtheria toxin. These observations demonstrated that a Tox^- strain might carry all or a portion of the *tox* gene cryptically. However, the methods for establishing this relationship were far too cumbersome to encourage an assessment of *tox* gene carriage by other Tox^- isolates. Recently we and others (3, 5) reported the isolation of a DNA restriction fragment of beta-converting phage which contains the gene for diphtheria toxin. The presence of the *tox* gene within this fragment has been confirmed by nucleotide sequencing, and segments of the fragment that can be used as probes for the *tox* gene have been

identified. Their availability has made it possible to determine in an efficient manner whether Tox^- strains carry DNA homologous to the *tox* gene.

MATERIALS AND METHODS

Bacteria and phages. *C. diphtheriae* strains C7(−), C7(β)$^{tox^+}$, C7(γ)$^{tox^-}$, and C7(β-*tsr*-3)$^{tox^+}$, a heat-inducible mutant of β used as a source of phage DNA, were taken from our stock collection. Similarly the six respiratory and six cutaneous isolates of toxinogenic *C. diphtheriae* and the single aerobic skin coryneform used in validating the *tox* probes were also taken from our collection and are identified below. Forty-three nontoxinogenic *Corynebacterium* spp. spanning roughly a 5-year period from 1976 through 1980, were sent to us from the Centers for Disease Control (CDC), Atlanta, Ga., by R. E. Weaver. Nineteen isolates were from South Dakota, six were from California, four were from Florida, three were from Alaska, and one each was from Montana, Nebraska, North Carolina, Hawaii, Massachusetts, Tennessee, Indiana, Canada, New Zealand, South America, and the Virgin Islands. A single toxinogenic isolate from South Dakota was also obtained from the CDC. All isolates were submitted to a series of tests routinely used in clinical laboratories to identify *C. diphtheriae*. With the exception of two isolates that utilized starch and glycogen and conformed to the criteria for *C. diphtheriae* var. *gravis*, the isolates conformed to the colonial morphology and physiological activities of *C. diphtheriae* var. *mitis* and closely related *Corynebacterium belfanti*. They all fermented glucose, maltose, and fructose and failed to act on lactose or sucrose. All were H_2S positive; 36 of 43 reduced nitrate (*C. diphtheriae*), whereas 7 failed to reduce nitrate (*C. belfanti*).

Media. Bacteria were grown in tryptose-yeast extract broth containing 10 g of tryptose, 5 g of yeast extract, and 5 g of Nacl per liter or in heart infusion broth (Difco Laboratories, Detroit, Mich.). The agar media contained 1.5% agar. In some cases Tween 80 (polyoxyethylene sorbitan mono-oleate; Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 0.2%. Deferrated Casamino Acids-yeast extract medium was prepared as described by Pappenheimer et al. (15).

Test for toxin production and activity. The modified gel immunodiffusion test for toxin production (12) was used to detect in vitro production. The rabbit intracutaneous test (1) was used to detect toxinogeny, i.e., the biological activity of toxin. ADP-ribosylating activity was determined by the method of Chung and Collier (4). The [^{14}C]NAD was purchased from Amersham Corp. (St. Louis, Mo.). Antitoxin was purchased from the Connaught Laboratories Ltd. (Ontario, Canada).

Genotype and phenotype designations for toxin. The gene for diphtheria toxin is *tox*. The wild-type gene is *tox*$^+$; a mutant gene is *tox*$^-$. To identify isolates in which there is no trace of the gene as determined by hybridizations with *tox* probes, we have employed the designation *tox*^{0}. The various phenotypes for *tox* are designated as follows: Tox, for biological activity of toxin as determined by animal inoculation; Crm, for production of cross-reacting material as determined by gel immunodiffusion tests with diphtheria antitoxin; and Adp, for the presence of ADP-ribosylating activity. In each case a positive response will be indicated by a ($^+$) and a negative response by a ($^-$). Where germane, more than one phenotype will be designated.

Bacterial DNA extractions. To extract bacterial DNA, cells grown overnight on heart infusion–Tween 80 agar were inoculated in 200 ml of heart infusion–Tween 80 broth and grown overnight with aeration. Cells were harvested by centrifugation and washed in an equal volume of distilled water. The pellet was suspended in 20 ml of 2.5 mM Tris (pH 8.0)–2.5 mM disodium EDTA–0.5 M sucrose–5 mg of hen egg white lysozyme (Calbiochem, La Jolla, Calif.) per ml. The cell suspension was incubated for 2 h at 35°C. The cells were harvested by centrifugation, and the pellet was suspended in 10 ml of 5 mM Tris (pH 7.4) containing 5 mM EDTA. Sodium dodecyl sulfate was added to a final concentration of 1% and mixed well. The cell suspension was heated to 50°C for 30 min to give complete lysis. Released DNA was sheared by repeated pipetting (15 to 20 times) of the suspension through a 10-ml pipette to produce the preparation termed "crude" bacterial DNA. Purified DNA was prepared as described by Schiller et al. (17), except that after RNase treatment, a phenol-chloroform (1:1) extraction preceded extraction with chloroform-isoamyl alcohol.

Phage DNA extraction. Beta phage DNA was prepared by the method previously described using phage from heat-induced C7(β-*tsr*-3)$^{tox^+}$ as the source (2).

Preparation of the *tox* probes. Purified β phage DNA was digested with *Eco*RI and *Xba*I restriction endonucleases simultaneously under conditions prescribed by *Eco*RI. After digestion, the preparation was electrophoresed overnight at 40 V in a preparative horizontal gel (1% agarose) to which ethidium bromide had been added. The gel was cut with a razor blade behind and ahead of the *tox*-containing band, and the DNA was electrophoresed for 1 h at 75 V into DEAE membrane paper (S S; NA-45) placed in the incision just ahead of the band of interest. This procedure follows the instructions supplied with the paper (S S; Sequences no. 364). The DNA was eluted with a high-salt solution containing 1 M NaCl, 0.1 mM EDTA, and 20 mM Tris (pH 8.0), precipitated twice, once from ethanol and once from isopropanol, and stored dry or in 5 mM Tris (pH 7.4) containing 0.5 mM EDTA. At this point the preparation was suitable for nick translation and use as the "A-B" probe or for further processing for the production of the "A" and "B" probes. To produce the A and B probes the A-B preparation was digested with *Mbo*I and processed as described above to isolate the two fragments.

Analytical procedures. The methods employed for restriction enzyme digests, agarose gel electrophoresis, nick translation with ^{32}P-deoxyribonucleotides, Southern blots, and autoradiography were described previously (2).

Dot-blot hybridizations. Either crude or purified DNA (2.5 to 5 μg) in 5- or 10-μl volumes were spotted on nitrocellulose filters (S S; BA 850, 0.45 μm) by using a 96 well template (S S; Minifold TM). When the samples were air dry, the filter was floated on a minimal amount (5 to 10 ml) of 0.5 N NaOH for 10 min. The filter was washed three times, 10 min each, with 1 M Tris (pH 7.4) containing 1.5 M NaCl, after which it was air dried and baked in vacuo at 80°C for 2 to 3 h.

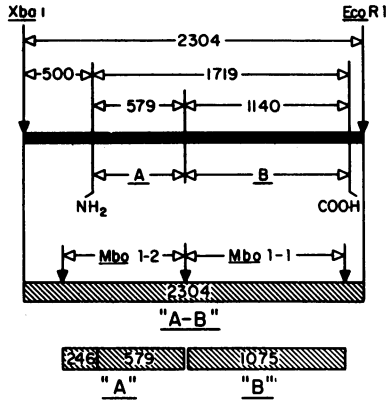

FIG. 1. Probes for the diphtheria toxin gene. The *Eco*RI-*Xba*I fragment is a 2.3-kilobase subfragment of the 3.6-kilobase *tox*-bearing *Bam*HI C fragment described by Buck and Groman (3). The isolation of this fragment and of the *Mbo*I fragments is described in the text. The internal *Mbo*I site is at the junction of the A and B coding sequences.

Subsequent steps in the hybridization procedure were the same as for Southern blots.

RESULTS

***tox* gene probes.** On the basis of our data identifying the *Bam*HI C fragment as the putative *tox*-bearing fragment of beta-converting phage DNA, Bjorn, Kaplan, and Collier (personal communication) determined the nucleotide sequence of the fragment and confirmed the presence of the *tox* gene. The relationship of certain segments of this fragment to the *tox* gene and the toxin molecule have been established, and probes for *tox* have been identified (Fig. 1). It is clear from Fig. 1 that the B probe, which contains over 90% of the sequence coding for B, is an unambiguous *tox* probe and also unambiguous for the B portion of the *tox* gene. The A probe is unambiguous in that it contains DNA coding for the A and not the B portion of the toxin molecule, but is ambiguous in that it contains approximately 250 base pairs in addition to those coding for the A fragment. The A-B probe contains the ambiguity of non-toxin-coding sequences at both ends.

The gel in Fig. 2 shows the relationship of the *tox*-bearing fragments to one another in various restriction enzyme digests. Because of the high concentration of agarose employed to demonstrate these relationships on a single gel, the first two fragments in the *Bam*HI digest are compressed. The *tox*-bearing fragment is the *Bam*HI C fragment previously described (3). The *Mbo*I fragments in lane C result from digestion of the *Eco*RI-*Xba*I *tox*-bearing fragment in lane B.

Validation of the *tox* probe. The dot-blot method was used to screen isolates of *C. diphtheriae* for the presence of DNA homologous to the *tox* gene. Bacterial DNAs were extracted, samples were spotted, denatured, and fixed to a nitrocellulose filter, and hybridization was carried out with ^{32}P-labeled *tox*-containing probes. An autoradiograph of a test of purified DNAs from a series of isolates is given in Fig. 3, and the results are summarized in Table 1.

The diversity of responses expected is illustrated by the results with the three C7 bacterial isogens. The parental C7(−) strain is negative on both the gel immunodiffusion (Elek) test and with the probes. The toxin-producing strain C7(β) is positive in both tests, and the non-toxin-producing strain C7(γ), for which there is independent evidence for carriage of the *tox* gene (see above), gave a negative gel immunodiffusion test, but was positive with the probes. The overall results show that all isolates of *C. diphtheriae* that gave a positive test by the gel immunodiffusion (Elek) test also gave a positive test with the *tox* DNA probes under conditions of high stringency. Included among these isolates were representatives of the three colonial types of *C. diphtheriae*, respiratory tract isolates collected over 30 years ago, and isolates from cutaneous sites which were collected within the

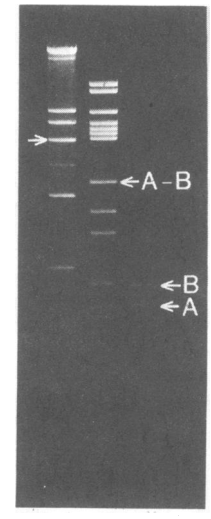

FIG. 2. Identification of restriction fragments utilized in probing for the diphtheria toxin gene. DNA from beta phage recovered from heat-induced C7(β-*tsr*−3)$^{tox^+}$ was extracted, purified, cut with restriction enzymes, and submitted to agarose (1.5%) gel electrophoresis. Lanes: A, *Bam*HI digest; B, simultaneous digest with *Eco*RI and *Xba*I; C, *Mbo*I digest of the *tox*-containing *Eco*RI-*Xba*I fragment. The arrows point to fragments containing elements of the *tox* gene. In the *Eco*RI-*Xba*I digest, the arrow identifies the A-B probe, and the *Mbo*I fragments A and B are the A and B probes defined in the legend to Fig. 1.

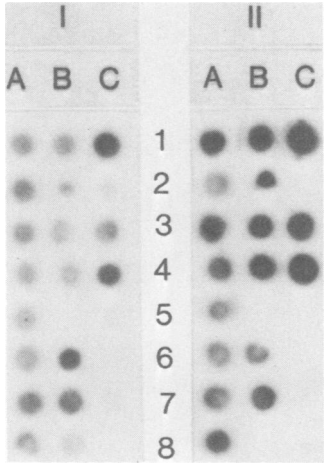

FIG. 3. Probe of *C. diphtheriae* for DNA homologous to the gene for diphtheria toxin. DNAs extracted from various isolates of *C. diphtheriae* and a single aerobic coryneform from the skin were hybridized with ^{32}P-labeled probes A-B (I) and B (II) (Fig. 1a) under conditions allowing for a mismatch of ca. 10% and autoradiographed. The strain designations and a summary of the results are given in Table 1.

last decade. Some of the isolates which were negative by the gel immunodiffusion test gave negative tests with the probes, but some gave positive tests. The three isolates exhibiting this latter behavior were from a series of nontoxinogenic isolates sent to us from the CDC for which more data will be presented later.

A close examination of Fig. 3 shows that there is an apparent discrepancy in some of the results given by the A-B and B probes. A number of isolates (Fig. 3; lanes B, isolate 8; lanes C, isolates 2, 5, 6, and 7) gave a weak positive reaction with the A-B probe, but were negative with the B probe. When C7(−) DNA was used as competing DNA in the A-B probe test, all of the weak reactions disappeared, and both probes gave the same results. It seems likely from this and other data that the A-B probe is contaminated with bacterial DNA fragments, and that this can be minimized or overcome by treating the intact phage preparation with DNase before extracting phage DNA. There is the possibility that some of these reactions with the A-B probe reflect homology of the bacterial genome with the ca. 600 base pairs that are outside the region coding for the *tox* structural gene. In recent hybridizations the A probe gave results identical with those given by the B probe. This indicates that if there is homology between the A-B probe and some chromosomal elements the sequences lie outside the regions encompassed by these two probes.

Probe of nontoxinogenic isolates. Forty-three isolates of nontoxinogenic corynebacteria were obtained from the CDC in Atlanta. On the basis of the tests we performed (see above), 36 isolates were designated *C. diphtheriae* var. *mitis* and seven were designated *C. belfanti*. DNA was extracted from all 43 isolates and hybridized with *tox* probes. The results for seven of these isolates with the A-B and B probe are shown in Fig. 3, lane C, and Table 1, section C. Of the 43 cultures, 29 gave the negative tests with the B probe and were also negative on the gel immunodiffusion test. However, 14 isolates that were negative by the gel immunodiffusion test, i.e., Crm$^-$, gave a positive test with the B probe when hybridizations were carried out under conditions of high stringency. Of the 14 isolates carrying *tox*-related DNA, 11 were isolated in South Dakota, and the other three were isolated from Montana, Florida, and Alaska. All were *C. diphtheriae* except the Florida isolate (778), which was a *C. belfanti* strain.

The relationship of these nontoxinogenic, *tox*-bearing isolates to one another was examined by comparing the restriction enzyme digest patterns of their genomic DNAs. DNAs extracted from all 14 isolates were digested with *Bam*HI and *Eco*RI and submitted to agarose gel electrophoresis (Fig. 4). All 11 South Dakota isolates, six of which are shown in Fig. 4 (lanes A, B, and D through G), and the Montana isolate (lane C), gave virtually identical patterns with *Bam*HI. The unique bands at the top of the gel in lane E are unexplained. Efforts to demonstrate plasmid DNA in this isolate were unsuccessful. The isolates in this group are designated as belonging to restriction pattern I (R-I). The isolates from Florida (Fig. 4, lane H) and Alaska (Fig. 4, lane I) are designated as belonging to the R-II and R-III restriction pattern groups, respectively. They had patterns uniquely distinct from that of the R-I group and from each other. Identical groupings were derived from the patterns produced with *Eco*RI. Thus, it appears that most of the *tox*-bearing isolates belonged to a common epidemiological cohort. However, it was clear that carriage of the *tox* gene by nontoxinogenic *C. diphtheriae* was not limited to one geographic site, nor was the origin of these strains likely to have been due to a single, unique event.

Additional tests for *tox* gene expression. All 14 isolates carrying *tox*-related DNA and failing to produce toxin-related material detectable by gel immunodiffusion (Crm−) were tested for toxinogeny by intracutaneous inoculation of a rabbit. All 14 were negative (Tox−) by this method. These same isolates were grown in deferrated Casamino Acids-yeast extract medium. The supernatants were concentrated 100-fold by $(NH_4)SO_4$ precipitation, and after dialysis the preparations were tested for ADP-ribosylating

TABLE 1. Isolates of *C. diphtheriae* in Fig. 1 probed with *tox* A-B and B[a]

A[b]					B[b]					C[b]				
No.[c]	Isolate no.	Colony type[d]	*tox* tests[e]		No.	Isolate no.	Colony type	*tox* tests		No.	Isolate no.	Colony type	*tox* tests	
			Elek	Probes				Elek	Probes				Elek	Probes
1	1100[f]	G	+	+	1	S136[g]	M	+	+	1	642[f]	M	−	+
2	1140[f]	M	+	+	2	S25[g]	I	+	+	2	643[f]	M	−	−
3	1250[f]	I	+	+	3	S620[g]	G	+	+	3	644[f]	M	−	+
4	1296[f]	I	+	+	4	S136[g]	M	+	+	4	645[f]	M	−	+
5	1341[f]	M	+	+	5	S615[g]	NA	−	−	5	648[f]	G	−	−
6	1200[f]	G	+	+	6	C7(β)[f]	M	+	+	6	649[f]	M	−	−
7	S414[g]	G	+	+	7	C7(γ)[f]	M	−	+	7	654[f]	M	−	−
8	S179[g]	G	+	+	8	C7(−)[f]	M	−	−	8	CT[h]	NA	NA	−

[a] All isolates are *C. diphtheriae* except S615, which is an aerobic skin coryneform.
[b] Indicates lane in Fig. 3.
[c] Refer to isolate numbers in Fig. 3.
[d] G, Gravis; M, mitis; I, intermedius; NA, colonial designation not applicable.
[e] Elek, Gel immunodiffusion test; probes, probes A-B and B. Both probes gave the same results (Fig. 1).
[f] Respiratory tract isolate.
[g] Cutaneous isolate.
[h] CT, Calf thymus DNA.

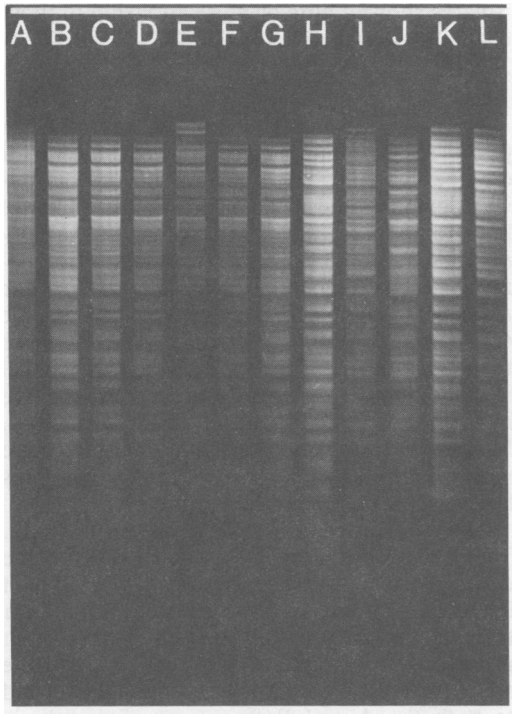

FIG. 4. Restriction enzyme digest patterns of DNA from *tox*-bearing, nontoxinogenic isolates of *C. diphtheriae*. DNA was purified from each isolate, digested with *Bam*HI, and electrophoresed in 1% agarose. The genetic and stock designations of the isolates are as follows: A through I (*tox*$^-$, Crm$^-$), (A) 642, (B) 653, (C) 774, (D) 800, (E) 801, (F) 804, (G) 805, (H) 778, (I) 787; lanes J and K (*tox*$^+$, Crm$^+$), (J) 798, (K) C7(β); and lane L (*tox*^{0}), C7(−).

activity. All 12 of the *tox* gene carriers in the R-I restriction group produced significant amounts of activity, but the R-II and R-III isolates were negative.

Location of the *tox* gene in *tox*-bearing isolates. Southern blots of restriction enzyme digests of chromosomal DNA of all 14 *tox*-bearing isolates were hybridized with both the A and B probes at high stringency. Representative results of *Bam*HI digests with the B probe are shown in Fig. 5. The same fragment of all the R-I isolates, seven of which are shown in Fig. 5 (lanes A through G), hybridized with the B probe and also with the A probe (data not shown). Similar results were obtained with the *Eco*RI digests. The fragments of the R-II and R-III isolates (Fig. 5, lanes H and I) that hybridized to probes A and B were at different positions, but again both probes hybridized to the same fragments. The *tox* gene in a toxin-producing culture isolated in South Dakota (Fig. 5, lane J) and that of control C7(β) were located in similar-sized fragments. These results strongly indicate that the entire *tox* gene is present and intact in these isolates. They also suggest that the *tox* gene may be inserted into different sites in different isolates, although it is possible that these differences reflect altered restriction sites.

Relation of *tox* gene carriage to beta phage carriage. The relationship of *tox* gene carriage to β and β-related corynebacteriophages is well established (10). To examine this further we have probed dot-blots of crude DNA from all 43 nontoxinogenic isolates obtained from the CDC with ^{32}P-labeled β phage. As expected, all 14 *tox*-bearing isolates gave positive reactions, but in addition five isolates not carrying the gene also gave positive reactions. Restriction enzyme digests of the genomic DNA of all 19 isolates were hybridized with ^{32}P-labeled β phage DNA. A representative selection of these results is shown in the autoradiograph in Fig. 6. Summarizing all the results, the data show that all 12 *tox*-bearing isolates of the R-I group gave identi-

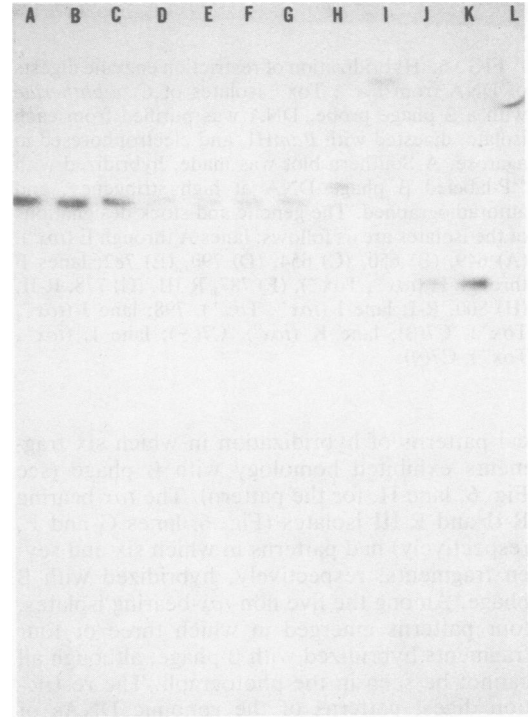

FIG. 5. Location of the *tox* gene in *Bam*HI digests of DNA from nontoxinogenic *C. diphtheriae*. A Southern blot of the agarose gel illustrated in Fig. 4 was hybridized with ^{32}P-labeled *tox* probe B at high stringency and autoradiographed. The genetic and stock designation of the isolates are as follows: lanes A through I (*tox*$^-$, Crm$^-$); (A) 642, (B) 653, (C) 774, (D) 800, (E) 801, (F) 804, (G) 805, (H) 778, (I) 787; lanes J and K (*tox*$^+$, Crm$^+$); (J) 798, (K) C7(β); and lane L (*tox*^{0}), C7(−). Note that the smudge in lane L is an artifact of the autoradiograph.

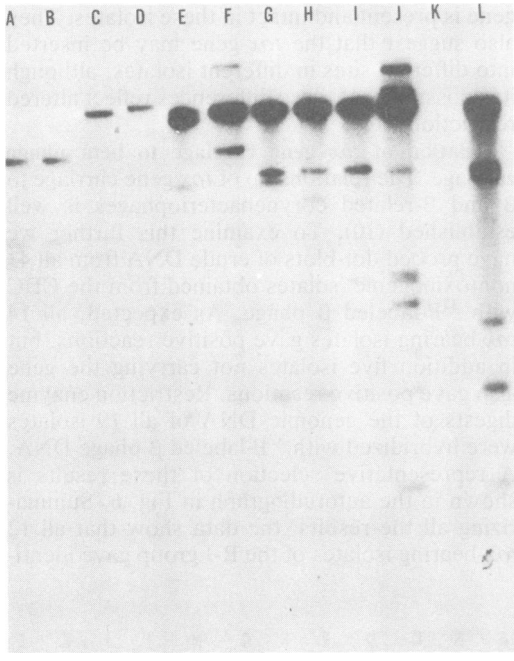

FIG. 6. Hybridization of restriction enzyme digests of DNA from *tox*$^-$, Tox$^-$ isolates of *C. diphtheriae* with a β phage probe. DNA was purified from each isolate, digested with *Bam*HI, and electrophoresed in agarose. A Southern blot was made, hybridized with ^{32}P-labeled β phage DNA at high stringency, and autoradiographed. The genetic and stock designations of the isolates are as follows: lanes A through E (*tox*^{0}), (A) 649, (B) 650, (C) 654, (D) 790, (E) 782; lanes F through H (*tox*$^-$, Tox$^-$), (F) 787, R-III, (G) 778, R-II, (H) 800, R-I; lane I (*tox*$^+$, Tox$^+$), 798; lane J (*tox*$^+$, Tox$^+$), C7(β); lane K (*tox*^{0}), C7(−); lane L (*tox*$^+$, Tox$^-$), C7(γ).

cal patterns of hybridization in which six fragments exhibited homology with β phage (see Fig. 6, lane H, for the pattern). The *tox*-bearing R-II and R-III isolates (Fig. 6, lanes G and F, respectively) had patterns in which six and seven fragments, respectively, hybridized with β phage. Among the five non-*tox*-bearing isolates, four patterns emerged in which three or four fragments hybridized with β phage, although all cannot be seen in the photograph. The restriction digest patterns of the genomic DNAs of these five isolates are distinct from those of the *tox*-bearing isolates or from each other, except that those of isolates 649 and 650 were identical. There were significant similarities in the β-hybridization patterns of the *tox*-bearing isolates (Fig. 6, lanes F through I) with those of both C7(β) and C7(γ); in the case of the one nontoxinogenic South Dakota isolate (Fig. 6, lane G), the pattern was identical to that of C7(γ). The β-hybridization patterns of the non-*tox*-bearing isolates showed little or no relationship to those of the *tox*-bearing cultures.

DISCUSSION

We have demonstrated the efficacy of DNA probes for detecting the presence of the gene for diphtheria toxin. The utility of the *tox* probes was demonstrated in dot-blot hybridizations of both toxinogenic and nontoxinogenic *C. diphtheriae* and in hybridization with restriction enzyme digest fragments of genomic DNAs. The *tox* gene was detected in a variety of toxinogenic *C. diphtheriae* collected over a span of more than 30 years. In every case a positive gel immunodiffusion test was matched by positive hybridization carried out at high stringency. Most nontoxinogenic organisms were negative with *tox* probes, but some were positive. This latter group was the focus of interest.

Three *tox* probes were employed in our study. The A-B probe contained sequences for the entire *tox* gene which represented about 75% of its base pair content. This probe did not appear to have absolute specificity for the *tox* gene, but proved useful as a screening probe. It had the advantage that it could detect sequences related to both the A and B fragments of diphtheria toxin simultaneously. Furthermore, it was much easier to prepare than the other probes, since a second restriction enzyme digestion and separation was necessary in the preparation of probes A and B. The A and B probes were useful in discriminating between sequences coding for the A and B fragments of toxin. The B probe was unambiguous in its detection of the B coding sequences, since it was internal to the B region and coded for over 90% of fragment B. However, the A probe contained approximately 250 base pairs proximal to the amino terminus of the A coding sequence. It has been shown by Smith et al. (18) that diphtheria toxin is a secretory protein and probably contains a leader sequence with a molecular weight of ca. 6,000. If the leader sequence is immediately proximal to the coding sequence for the amino terminus as indicated by their data, one can calculate that it would take approximately 160 of the 250 base pairs to code for it. Thus, most, if not all, of the A probe is specific to toxin protein synthesis. In addition to this one might expect that sequences concerned with the regulation of toxin synthesis would also be present and precede the leader sequence. The specificity of such regulatory sequences to the *tox* gene might be questioned since there is substantial evidence that regulation of toxin synthesis is under at least partial control by host genes (13). However, our evidence suggested that all of the sequences in the A probe were specific for the *tox* operon. Thus, the A probe hybridized to only a single fragment

in digests of genomic DNA of a number of *tox*-bearing isolates, and in all cases this was the same fragment to which the B probe hybridized. Therefore, the ca. 250 base pairs that lie proximal to the sequences that code for the A fragment of diphtheria toxin seem linked specifically to that gene. The fact that the A probe did not hybridize to any fragment in digests of C7(−) genomic DNA reinforces this conclusion.

Examination of nontoxinogenic isolates obtained from the CDC revealed that 14 of 43 isolates contained the *tox* gene, or at least major elements of the A and B coding regions. The characteristics of these isolates are summarized in Table 2. Twelve of the isolates (11 from South Dakota and 1 from Montana, an adjacent state) appeared to be identical and had been collected roughly over a 4-year period (1976 through 1980). They shared a common restriction enzyme digest pattern for genomic DNA, a common site for the *tox* gene and a common phenotype (Tox^-, Crm^-, Adp^+) in relation to *tox* gene expression. The fact that they comprised a single epidemiological cohort bears on the interpretation of the frequency with which *tox*-bearing Tox^- strains arise. Thus, they arise less frequently than the raw data make it appear. Nevertheless, it seems clear that *tox*-bearing, Tox^- strains have arisen more than once, i.e., in independent events. The isolates from Alaska and Florida are very different from the South Dakota cohort in both their genomic digest patterns and in the absence of ADP-ribosylating activity in culture supernates, and the prototype *tox*-bearing Tox^- strain C7(γ), also has a unique restriction digest pattern.

The isolation of additional tox^-, Tox^- *C. diphtheriae* demonstrates emphatically that nontoxinogenic strains represent a potential reservoir for the *tox* gene and for the possible reemergence of toxinogeny. It has been shown experimentally (12) that two tox^- converting phage mutants can recombine to produce tox^+ converting phage. The fact that the South Dakota isolates were present at least over a period of roughly 4 years also shows that carriage of a mutant *tox* gene is not necessarily a short-lived relationship. Rather it shows that under some natural conditions the gene can be retained for a number of years. We have maintained C7(γ) in our laboratory for over 30 years without detectable change in the status of its *tox* gene, and at a minimum this indicates the stability of the prophage-host relationship and of the *tox* gene within the phage genome.

Very little is known about factors contributing to the survival or selection (or both) of toxinogenic or nontoxinogenic *C. diphtheriae*. A priori one would postulate that toxinogenic organisms would be favored in a nonimmune population, whereas nontoxinogenic organisms would be favored in immune populations. Pappenheimer (14) cites unpublished evidence from Maximescu favoring this hypothesis. The effect of nonexpression or partial expression of the *tox* gene on selection is also an interesting question, one for which many outcomes could be imagined. In the simplest formulation one would anticipate that partial expression would be a drain on the strain's economy and would be selected against more actively than nonexpression. The fact that we have found both partially expressed and unexpressed *tox* genes in natural isolates does not necessarily invalidate this reasoning. We simply do not know enough about selective conditions to make a critical analysis.

There are probably a variety of mechanisms by which *tox* gene expression is completely shut off or allowed partial expression. In the case of C7(γ), lack of expression is probably due to insertion of a piece of bacterial DNA early in the structural gene or in its regulatory region (3). Other mutational events such as point mutations, deletions, or inversions can also be invoked to explain the various phenotypes. Mutants have been produced artifically by nitrosoguanidine treatment of β-converting phage with phenotypes identical to those found naturally in the tox^-, Tox^- isolates (9, 12, 19). In addition to mutation in phage genes, it is clear that host gene mutations may also be responsible for variation in the expression of the *tox* gene (11). The extent of these interactions is a topic of continuing study.

TABLE 2. Summary of the characteristics of the nontoxinogenic, *tox*-bearing isolates of *C. diphtheriae*.

Isolate no.	Origin	Restriction pattern[a]	Phenotype[b]		
			Crm	Tox	Adp
642	S. Dak.	I	−	−	+
644	S. Dak.	I	−	−	+
645	S. Dak.	I	−	−	+
646	S. Dak.	I	−	−	+
647	S. Dak.	I	−	−	+
652	S. Dak.	I	−	−	+
653	S. Dak.	I	−	−	+
774	Mont.	I	−	−	+
778	Fla.	II	−	−	−
787	Alaska	III	−	−	−
800	S. Dak.	I	−	−	+
801	S. Dak.	I	−	−	+
804	S. Dak.	I	−	−	+
805	S. Dak.	I	−	−	+

[a] Based on patterns in agarose gels after digestion with *Bam*HI or *Eco*RI.

[b] Crm, by gel immunodiffusion test; Tox, by rabbit intradermal test; Adp, by test for ADP ribosylation (see the text).

Finally, we come to the question of the relationship between toxinogeny in *C. diphtheriae* and bacteriophage. We have observed that β-related phage DNA is present in all the *tox*-bearing, nontoxinogenic *C. diphtheriae* and in the one *tox*-bearing, nontoxinogenic *C. belfanti* isolate, as well as in some isolates lacking a *tox* gene. However, the former seem to be more closely related to β than the latter. The strong correlation between β-like phage DNA and *tox* carriage in nontoxinogenic *C. diphtheriae* suggests, but does not prove that the *tox* gene is phage associated in these isolates. If this is the case, it would suggest that even when the *tox* gene is nonfunctional as a component of virulence, its association with phage persists. In principle it is not possible to ever determine whether the *tox* gene exists independently of some phage. However, with the technique available, it may be possible to determine whether the *tox* gene can be found independently of β-related phages.

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