JOURNAL OF BACTERIOLOGY, Dec. 1978, p. 1135-1142
0021-9193/78/0136-1135$02.00/0

Vol. 136, No. 3
Printed in U.S.A.

Isolation and Characterization of Extragenic Suppressor Strains of *Corynebacterium diphtheriae*

P. BACHA* AND J. R. MURPHY

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

Received for publication 1 September 1978

The isolation and characterization of two different nonsense suppressor strains of *Corynebacterium diphtheriae* C7 *sup*$^+$(−)$^{tox-}$ are described. Appropriate lysogens of these strains with corynephage β, carrying known class II *tox* premature polypeptide chain termination mutations [C7*sup-1*(β^{tox-30}) and C7*sup-2*(β^{tox-45})], each produce a 62,000-dalton polypeptide with nicotinamide adenine dinucleotide: elongation factor-2 adenosine diphosphate ribosyltransferase activity in addition to a chain-terminated polypeptide of 30,000 or 45,000 daltons, respectively. In addition, purified protein of 62,000 daltons, resulting from the suppression of the nonsense mutations *tox-30* and *tox-45*, will react with antisera purified against the terminal 17,000 daltons of the toxin molecule and are immunologically identical to toxin by radial immunodiffusion. The suppression pattern of lysogenic derivatives of C7*sup-1*(−)$^{tox^-}$ and C7*sup-2*(−)$^{tox^-}$ with other class II and III mutants of corynephage β was determined.

Corynebacteriophage β was shown to carry the structural gene for diphtheria toxin by isolating phage mutants which code for the production of nontoxic proteins that are serologically related to diphtheria toxin (23). Toxicity is dependent upon two functional domains of the toxin molecule: fragment A, which is enzymatically active and catalyzes the transfer of the adenosine diphosphoribosyl moiety (ADPR) from nicotinamide adenine dinucleotide (NAD) to eucaryotic polypeptide elongation factor-2 (EF-2), and fragment B, which binds the toxin molecule to specific receptors on the surface of sensitive eucaryotic cells. Mutations within either domain may greatly affect the specific toxicity. Nontoxic proteins, regardless of their enzyme activity, are referred to as cross-reacting material (CRM).

The β phage Tox$^-$ mutants isolated previously fall into three classes: (I) mutants that produce CRM of 62,000 molecular weight (15, 24); (II) mutants that produce CRM of less than 62,000 (11, 15, 24); and (III) CRM$^-$ mutants that do not produce detectable *tox* gene products (11, 15). Class I mutants are probably missense, whereas those of class II are most likely either nonsense or deletion mutants. The mutants in class III, on the other hand, may be nonsense, frameshift, or large deletions, or they may possibly result from mutations in an as yet unknown *tox* regulatory locus.

We have been interested in the regulation of diphtheria toxin production and have approached this problem by in vitro synthesis of diphtheria *tox* gene products by supernatants of cellular extracts of *Escherichia coli* spun at 30,000 × *g* for 30 min (18) and by isolation of mutant β phage lysogens of *Corynebacterium diphtheriae* that are partially *tox* constitutive (19). Recently, our attention was turned to the class III mutants since isolation of additional regulatory mutants has been hampered by an inability to classify CRM$^-$ mutants as either nonsense or potentially regulatory due to a lack of strains necessary for their genetic characterization.

The discovery of amber (UAG) (2), ochre (UAA) (3), and opal (UGA) (21, 25) suppressor (Sup$^-$) strains of *E. coli* has allowed the genetic characterization of conditional mutations by extragenic suppression of nonsense codons. The use of Sup$^-$ strains has become an integral part of the genetic characterization of mutants in several procaryotic and eucaryotic systems (16). To date, suppressor strains of *C. diphtheriae* have not been described. In this communication we report the isolation and characterization of two different Sup$^-$ strains of the nonlysogenic, nontoxigenic *C. diphtheriae* strain, C7*sup*$^+$(−)$^{tox^-}$.

MATERIALS AND METHODS

Bacterial strains. The bacteria used in this study are listed in Table 1. All lysogenic strains are deriva-

tives of the nonlysogenic *C. diphtheriae* C7*sup*$^+$ $(-)^{tox^-}$, which has been described previously (1).

Culture media. Bacteria were grown in either PT medium (19) for mutagenesis and phage induction or in low-iron CY medium (20) for optimal *tox* gene expression. Before use, 3 ml of 50% maltose–0.5% calcium chloride solution was added aseptically per 100 ml of medium. Plates were made by adding 1% agar (Difco Laboratories, Detroit, Mich.) to PT medium or 1% Noble agar (Difco) to CY medium before sterilization.

Mutagenesis and isolation of *C. diphtheriae* Sup$^-$ mutants. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich) was added to a final concentration of 60 μg/ml to a culture of the nonlysogenic, nontoxigenic *C. diphtheriae* strain C7*sup*$^+$$(-)^{tox^-}$ grown in PT medium to an optical density at 590 nm of 0.5. The cells were incubated for 1 h in the dark, washed, suspended in fresh medium, and incubated for 2 h at 34°C. Surviving bacteria were grown on PT agar plates that were incubated overnight. Individual colonies were then transferred to three separate square integrid petri dishes (Falcon 1012; Falcon Plastics, Oxnard, Calif.) containing CY agar. In each series, the first plate served as a nonlysogenic master. The second (prespread with 10^6 plaque-forming units of phage β^{tox-30}) and third (prespread with 10^6 plaque-forming units of phage β^{tox-45}) were used to screen for the respective lysogenic derivatives. After 24 h of incubation at 34°C, sterile strips of Whatman no. 1 filter paper (2 by 125 mm) saturated with diphtheria antitoxin (500 U/ml; lot SA10 purchased from the Massachusetts Department of Public Health, Jamaica Plain, Mass.) were placed between rows of colonies on the second and third plates of each series. The plates were incubated for an additional 24 h, at which time each colony was examined for a strong, sharp toxin precipitin band indicative of a CRM of 62,000 daltons. CRM of 30,000 or 45,000 daltons (CRM30 or CRM45) produce faint precipitin bands with the antisera used. Colonies that remained positive after retesting were cloned, and the enzymatic activities and molecular weights of the *tox* gene products were determined.

Production and purification of *tox* gene products. Cultures of the appropriate lysogens of *C. diphtheriae* were grown in 100-ml volumes of CY medium in 2-liter Erlenmeyer flasks for 18 to 24 h at 34°C with shaking at 240 rpm in a New Brunswick model VS shaker. The cells were centrifuged at 8,000 × *g* for 20 min at 5°C. The supernatant fluids were treated for 10 min at room temperature with 350 μg of phenylmethylsulfonylfluoride (Sigma Chemical Co., St. Louis, Mo.) per ml dissolved in 95% ethanol. Ammonium sulfate was then added to 50% saturation. The resulting precipitate was removed by centrifugation at 9,000 × *g* for 15 min and discarded. The supernatant was brought up to 75% saturation with ammonium sulfate and centrifuged. The precipitate was resuspended in 5 ml of 0.01 M sodium phosphate buffer, pH 7.2, dialyzed against phosphate buffer, and purified by ion-exchange chromatography on Whatman DE-52 essentially as described by Pappenheimer et al. (20).

Polyacrylamide gel electrophoresis. Samples of purified *tox* gene products were mixed with 25 μl of sample application buffer without β-mercaptoethanol and run in pairs on 10% polyacrylamide gels with 0.1% sodium dodecyl sulfate (SDS) for 4 h at 8 mA per tube in a Hoeffer model GT 6 apparatus, according to the procedure of Gill and Dinius (7). One of each set was stained with 0.1% Coomassie brilliant blue in methanol–acetic acid–water (5:1:5) for 2 h and destained overnight in a methanol–acetic acid–water mixture (0.1:0.75:10) with about 500 mg of Bio-Rex RG-5010-X8 20-50 mesh mixed-bed resin at 37°C in a roller drum. Stained gels were scanned at 550 nm in a Beckman model 25 spectrophotometer. The second gel of each set was sliced for the determination of NAD:EF-2 ADPR-transferase activity.

Determination of NAD:EF-2 ADPR-transferase activity. SDS-polyacrylamide gels were sliced into 2-mm disks. Each fraction was incubated overnight at 37°C in 200 μl of 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 8.0, containing 5 mM dithiothreitol. The entire extract was treated with 1 μg of trypsin at 37°C for 10 min, followed by 20 μg of soybean trypsin inhibitor, and was

TABLE 1. *Bacterial strains used*

Strains	Phenotype	Source	Reference
C7*sup*$^+$$(-)^{tox^-}$	CRM$^-$	A. M. Pappenheimer, Jr.	(1)
C7*sup*$^+$(β^{tox^+})	CRM$^+$	A. M. Pappenheimer, Jr.	
C7*sup*$^+$(β^{tox-30})	CRM$^+$	A. M. Pappenheimer, Jr.	
C7*sup*$^+$(β^{tox-45})	CRM$^+$	A. M. Pappenheimer, Jr.	
C7*sup*$^+$($\beta^{tox-111}$)	CRM$^+$	N. B. Groman	(15)
C7*sup*$^+$($\beta^{tox-112}$)	CRM$^+$	N. B. Groman	
C7*sup*$^+$($\beta^{tox-113}$)	CRM$^+$	N. B. Groman	
C7*sup*$^+$($\beta^{tox-116}$)	CRM$^-$	N. B. Groman	
C7*sup*$^+$($\beta^{tox-117}$)	CRM$^-$	N. B. Groman	
C7*sup*$^+$(β^{tox-1})	CRM$^+$	R. K. Holmes	(11)
C7*sup*$^+$(β^{tox-2})	CRM$^+$	R. K. Holmes	
C7*sup*$^+$(β^{tox-3})	CRM$^+$	R. K. Holmes	
C7*sup*$^+$(β^{tox-4})	CRM$^-$	R. K. Holmes	
C7*sup*$^+$(β^{tox-5})	CRM$^-$	R. K. Holmes	
C7*sup*$^+$(β^{tox-6})	CRM$^-$	R. K. Holmes	
C7*sup*$^+$(β^{tox-7})	CRM$^-$	R. K. Holmes	
C7*sup*$^+$(γ^{tox^-})	CRM$^-$	American Type Culture Collection	(9)

then assayed directly for transferase activity according to the procedure of Gill and Pappenheimer (8), substituting wheat germ EF-2 (4) for rabbit reticulocyte EF-2. The reaction was stopped after 1 h by adding cold 5% trichloroacetic acid. The resulting precipitate was collected on Whatman GF/A filters, washed, dried, and counted in Econofluor (New England Nuclear Corp., Boston, Mass.) in a Beckman liquid scintillation counter.

Antitoxin and suppressed protein purification. Diphtheria toxin and CRM45 were purified from cultures according to the procedure outlined above. These proteins were then separately attached to CNBr-activated Sepharose (6B; Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) according to the procedure of Cuatrecasas et al. (5). Diphtheria antitoxin was applied to the toxin-Sepharose column, which was washed with 0.2 M borate–0.2 M NaCl (pH 8.0) buffer. Highly purified antitoxin was eluted from the column with 0.05 M glycine-hydrochloride, pH 2.9, buffer. After dialysis against 0.2 M borate–0.2 M NaCl (pH 8.0) buffer, purified antitoxin was then applied to the CRM45-Sepharose affinity column. The protein in the initial wash-through contained antibody directed against only the C-terminal 17,000 daltons of the toxin molecule, because those directed against the N-terminal 45,000 daltons were bound to it. This antitoxin, designated AT-C17, will form a precipitin line against toxin but not against CRM45 in both radial immunodiffusion and counterimmunoelectrophoresis assays.

In turn, AT-C17 was attached to CNBr-activated Sepharose. Ammonium sulfate-precipitated *tox* gene products of C7*sup*-1 (β^{tox-30}) and C7*sup-2* (β^{tox-45}) were chromatographed on the AT-C17 affinity column in the manner described above. The CRM30 or CRM45 passed through the column, whereas normal-length suppressed protein that bound to it was eluted with the glycine buffer wash.

These purified proteins, designated *30-sup-1-62* and *45-sup-2-62*, respectively, were then used for radial immunodiffusion and counterimmunoelectrophoresis. In the latter case, they were subjected to electrophoresis against AT-C17 in 1% agarose in barbital buffer (0.02 M barbital–0.0135 M sodium acetate–0.0015 M sodium azide) for 4 h at 20 mA on a glass slide (8.3 by 10.1 cm). Plates from both procedures were pressed and dried before staining with 0.5% Coomassie blue in a 5:1:4 mixture of methanol–acetic acid–water. The same concentration of methanol–acetic acid–water was used to destain the plates.

RESULTS

Nontoxigenic mutants of corynephage β can be divided into three groups according to the nature of their diphtheria *tox* gene product. Class I mutants code for the production of CRM of 62,000 molecular weight that is serologically identical to diphtheria toxin, whereas the premature polypeptide chain termination products of class II mutants are only partially related. Recently, Holmes (11) and Laird and Groman (15) have described class III mutants of corynephage β that are serologically unreactive and therefore phenotypically CRM$^-$. Nonsense mutations within the *tox* structural gene could give rise to both class II and III mutants; however, the genetic characterization of these two groups of mutants has remained incomplete since extragenic suppressor (Sup$^-$) strains of *C. diphtheriae* have not been previously described.

One would predict that if the premature polypeptide chain termination mutations in either β^{tox-30} or β^{tox-45} were nonsense, then lysogenic derivatives of corynebacterial Sup$^-$ strains would synthesize normal-length toxin. With this in mind, we screened over 1,500 individual clones of the nontoxigenic, nonlysogenic C7*sup*$^+$(−)$^{tox-}$ strain of *C. diphtheriae* that had survived nitrosoguanidine mutagenesis for the ability to suppress the mutations in β^{tox-30} and β^{tox-45}. Two potential Sup$^-$ strains were isolated and designated C7*sup-1*(−)$^{tox-}$ and C7*sup-2*(−)$^{tox-}$.

Figure 1 shows the precipitin line formed by various lysogens of Sup$^+$ and Sup$^-$ strains of *C. diphtheriae* after growth on CY agar. As can be seen, C7*sup*$^+$(−)$^{tox-}$ and C7*sup*$^+$(β^{tox-30}) do not form a precipitin band. On the other hand, the band formed by C7*sup*$^+$(β^{tox-4b}) is weak and diffuse, whereas, those formed by C7*sup*$^+$(β^{tox+}), C7*sup-1*(β^{tox-30}), and C7*sup-2*(β^{tox-45}) are sharply defined. The antitoxin used in the test has been previously shown to be primarily directed against antigenic determinants in the C-terminal 17,000 daltons of the diphtheria toxin molecule (20) and therefore reacts most strongly against a protein of 62,000 daltons.

To rule out the possibility that reversion of the corynephage mutations in β^{tox-30} and β^{tox-45} were responsible for the precipitin band, UV-induced phage from these strains were plated on lawns of C7*sup*$^+$(−)$^{tox-}$ and lysogens were picked; these were reexamined. In all instances, the lysogens reacted as the parental strains and produced only weak, diffuse precipitin lines. Electrophoresis of the purified *tox* gene products of these lysogens resolved only CRM30 and CRM45, respectively.

Duplicate samples of the *tox* gene products expressed by C7*sup*$^+$(β^{tox-30}) and C7*sup-1*(β^{tox-30}) were purified and subjected to electrophoresis on SDS-polyacrylamide gels. One gel was stained with Coomassie blue, whereas the other was sliced into 2-mm fractions. The protein from these slices was eluted into fresh buffer, and then it was assayed for NAD:EF-2 ADPR-transferase activity. In Fig. 2A, the *tox* products expressed by C7*sup*$^+$(β^{tox-30}) migrate as a broad polypeptide band with the NAD:EF-2 ADPR-transferase activity and an electrophoretic mobility between 24,000 and 30,000 daltons. During initial purification steps, a proteolytic cleavage can occur separating fragment A (the

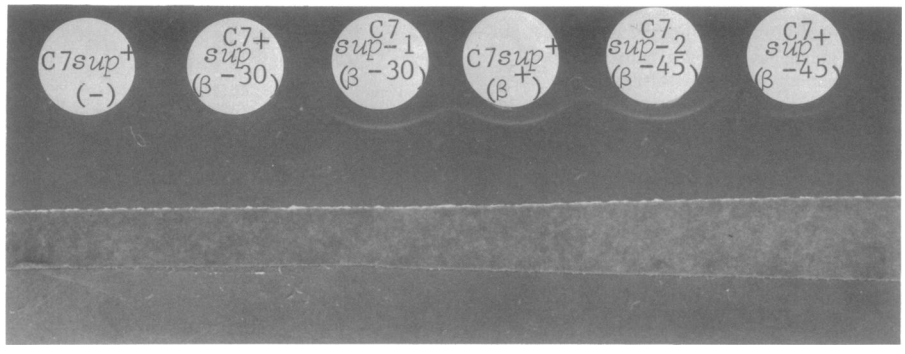

FIG. 1. *Test showing precipitin bands formed by wild-type C. diphtheriae both nonlysogenic and lysogenic for corynephage β^{tox+}, β^{tox-30}, and β^{tox-45}. For comparison, bands formed by a Sup-1 strain lysogenic for β^{tox-30} and a Sup-2 strain lysogenic for β^{tox-45} are shown.*

enzymatically active N-terminal 24,000 daltons) from the terminal 6,000 daltons. The absolute amount of nicking will vary from preparation to preparation. Therefore, a protein band at 24,000 daltons with enzyme activity and varying intensity may often be seen.

In contrast, in Fig. 2B the *tox* gene products expressed by C7*sup-1*(β^{tox-30}) migrate in three distinct polypeptide bands, each with NAD:EF-2 ADPR-transferase activity and mobilities corresponding to 62,000, 30,000, and 24,000 daltons. To see the band at 62,000, the gels had to be overloaded with protein, which broadened the other bands. Analogous results were obtained after the SDS-polyacrylamide gel electrophoresis of purified products of C7*sup*$^+$(β^{tox-45}) and C7*sup-2*(β^{tox-45}). Integration of the NAD:EF-2 ADPR-transferase activity peaks from the gels suggests that the level of suppression is approximately 8% in C7*sup-1*(β^{tox-30}) and 16% in C7*sup-2*(β^{tox-45}).

The immunological demonstration of extragenic suppression was made by purifying antitoxin directed against the C-terminal 17,000-dalton region of the diphtheria toxin molecule (AT-C17). Diphtheria toxin and the larger *tox* gene products expressed by C7*sup-1*(β^{tox-30}) and C7*sup-2*(β^{tox-45}) formed a precipitin line after counterimmunoelectrophoresis, whereas CRM30 and CRM45 were unreactive (Fig. 3). Figure 4 shows that the larger *tox* gene products produced by C7*sup-1*(β^{tox-30}) and C7*sup-2*(β^{tox-45}) are immunologically identical to diphtheria toxin on radial immunodiffusion plates.

Since we had demonstrated that the premature polypeptide chain termination mutations in β^{tox-30} and β^{tox-45} were partially suppressed to a 62,000-molecular-weight form with enzyme activity in the appropriate Sup$^-$ strain of *C. diphtheriae*, it was of interest to examine the potential suppression of other class II, as well as class III, *tox* mutants. The appropriate lysogenic derivatives of C7 were induced with UV light. The β phage released were sterilized by membrane filtration and plated separately on lawns of C7*sup-1*(−)$^{tox-}$ and C7*sup-2*(−)$^{tox-}$. Lysogenic derivatives were purified and grown under optimal conditions for *tox* expression. In each case, the *tox* gene products were purified by ammonium sulfate precipitation and ion exchange chromatography and were characterized according to NAD:EF-2 ADPR-transferase activity and apparent molecular weight on SDS-polyacrylamide gels (Table 2). In five additional cases, the mutations in *tox* resulting in class II and III phenotypes were suppressed, and the size of the *tox* gene product was restored to 62,000. Indeed, the suppression of the *tox* mutation was strain specific. In the case of the class III mutant β^{tox-4}, the apparent molecular weight of the *tox* product synthesized was dependent upon the host Sup$^-$ strain: the lysogen C7*sup-2*(β^{tox-4}) produced a *tox* gene product of 48,000 daltons, whereas C7*sup-1*(β^{tox-4}) produced one of 62,000.

DISCUSSION

There are two lines of evidence which strongly suggest that regulation of the corynephage β *tox* gene is mediated by a corynebacterial host factor. The first is the specific inhibition of *tox* expression by addition of C7*sup*$^+$(−)$^{tox-}$ extracts to an in vitro protein-synthesizing system consisting of *E. coli* cellular extracts programmed with β DNA (18), and the second is isolation of bacterial host mutants of *C. diphtheriae* that, as lysogens of β^{tox+}, are *tox* constitutive (13). Nonetheless, the isolation of corynephage mutants which do not produce detectable *tox* gene products raises the possibility that diphtheria *tox* expression may, in part, be regulated by an un-

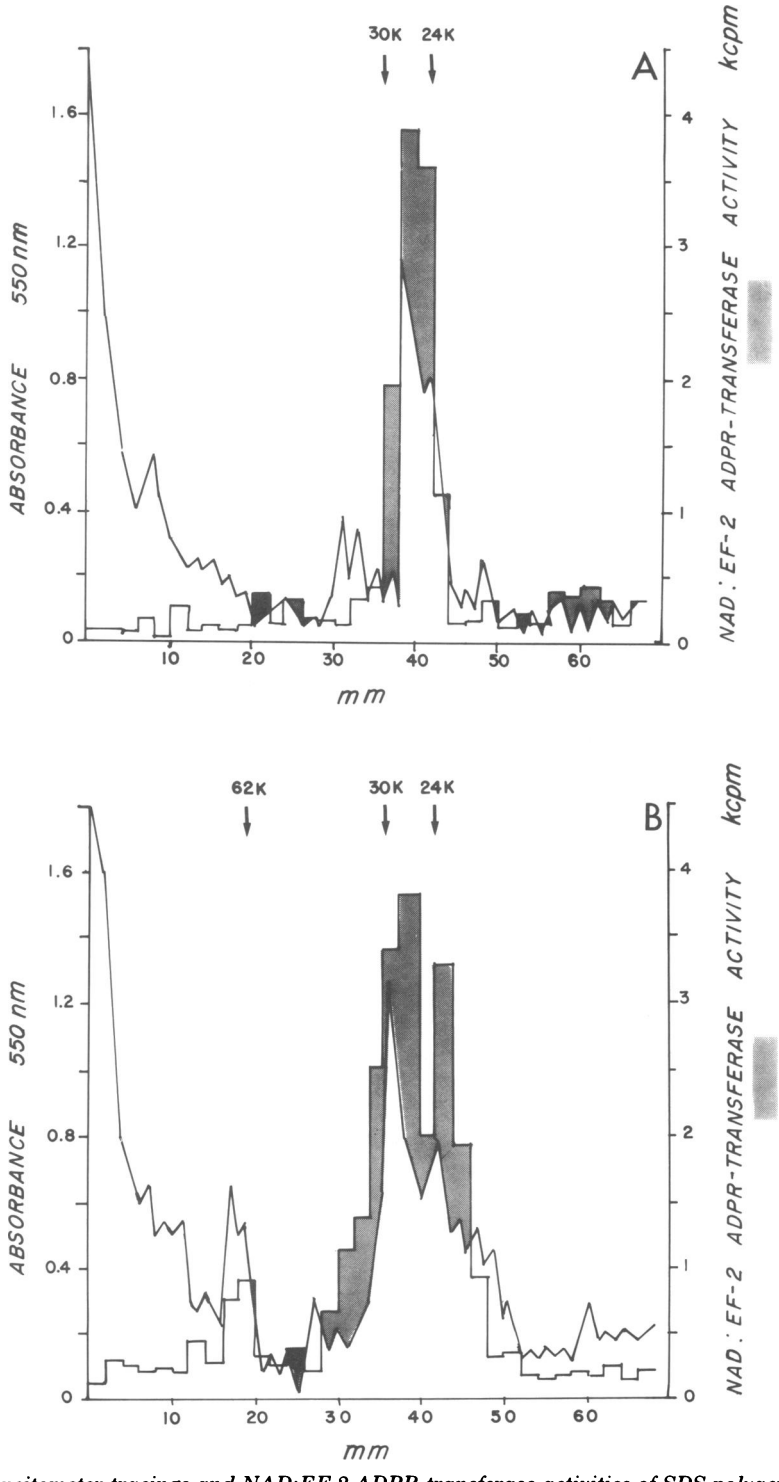

FIG. 2. *Densitometer tracings and NAD:EF-2 ADPR-transferase activities of SDS-polyacrylamide gels of purified tox gene products from (A) C7sup⁺(β^{tox-30}) and (B) C7sup-1(β^{tox-30}).*

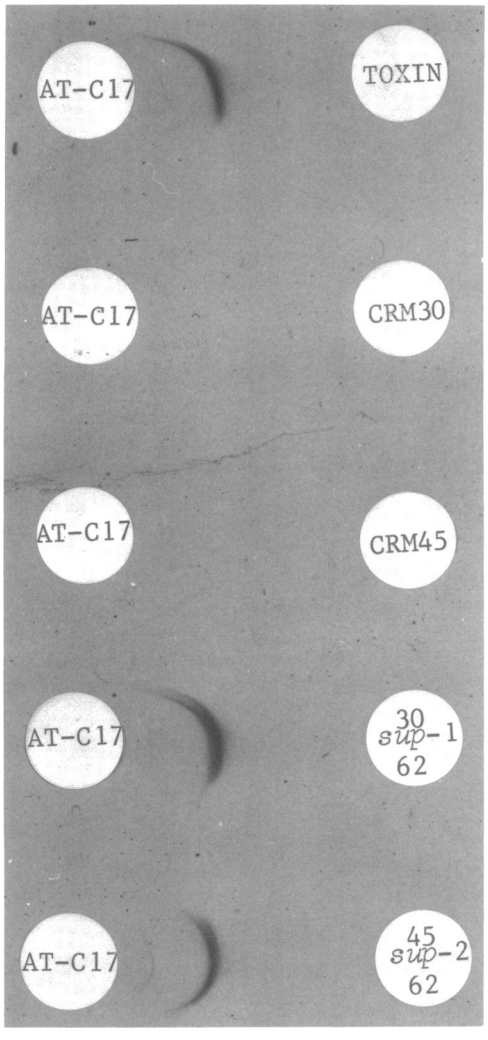

FIG. 3. *Counterimmunoelectrophoresis of various purified tox gene products against antitoxin directed against the terminal 17,000 daltons of the toxin molecule (AT-C17). The designations 30 sup-1-62 and 45 sup-2-62 refer to purified 62,000-molecular-weight protein isolated from culture supernatants of C7sup-1(β^{tox-30}), and C7sup-2(β^{tox-45}), respectively.*

known corynephage factor.

All of the CRM$^-$ mutants of corynephage β so-far described have been induced by nitrosoguanidine mutagenesis. Since nitrosoguanidine is known to give rise mainly to single base-pair substitution mutants that are either nonsense or missense (24), it is reasonable to believe that several of the mutations within the diphtheria *tox* gene that result in the CRM$^-$ phenotype may be nonsense. Extragenic suppressor (Sup$^-$) strains of *C. diphtheriae* have not been previously isolated. As a result, the genetic characterization of CRM$^-$ mutants, as well as *tox* premature polypeptide chain termination mutants, has remained incomplete.

We have described the isolation and partial characterization of two different extragenic suppressor strains of *C. diphtheriae*. We have used an immunological scoring technique for potential suppressor strains that relies upon antitoxin directed primarily against the C-terminal 17,000-dalton region of the toxin molecule. This antitoxin, lot no. SA10 from the Massachusetts Antitoxin and Vaccine Laboratory, has been shown to react strongly with diphtheria toxin, but to cross-react weakly with CRM45, and almost not at all with CRM30 (20).

From over 1,500 potential mutants examined, two stable Sup$^-$ strains of *C. diphtheriae* were independently isolated. These strains have been designated C7*sup-1*$(-)^{tox-}$ and C7*sup-2*$(-)^{tox-}$. Suppression of the two different nonsense mutations *tox-30* and *tox-45* in the appropriate lysogenic background [C7*sup-1*(β^{tox-30}) and C7*sup-2*(β^{tox-45})] each result in the production of a protein that migrated as a single band of 62,000 daltons with NAD:EF-2 ADPR-transferase activity in SDS-polyacrylamide gels and which was immunologically reactive against antitoxin that had been absorbed with CRM45. In addition, the larger-molecular-weight proteins produced by both C7*sup-1*(β^{tox-30}) and C7*sup-2*(β^{tox-45}) were immunologically identical to diphtheria toxin by radial immunodiffusion against unabsorbed antisera. The level of suppression in C7*sup-1*(β^{tox-30}) is approximately 8%, and it is 16% in the case of C7*sup-2*(β^{tox-45}).

Lysogenic derivatives of C7*sup-1*$(-)^{tox-}$ and C7*sup-2*$(-)^{tox-}$ were constructed with all previously described class II and III diphtheria *tox* gene mutants. In two additional instances, the class II mutations were suppressed in either one or both of the Sup$^-$ strains, and a 62,000-dalton protein was produced. In three class III mutations extragenic suppression was observed. As seen in Table 2, the suppression pattern of C7*sup-1*$(-)^{tox-}$ overlaps that of C7*sup-2*$(-)^{tox-}$. This may be explained, in part, by the observation that in *E. coli* ochre suppressors can recognize amber nonsense mutations as well as ochre mutations (11).

The isolation of two different Sup$^-$ strains of C7*sup*$^+(-)^{tox-}$ has allowed us to further characterize the class II and III *tox* mutants of corynephage β. The extragenic suppression of four of the premature polypeptide chain termination mutations shows that in many instances the class II mutations are nonsense. In addition, we have been able to separate the class III mutants

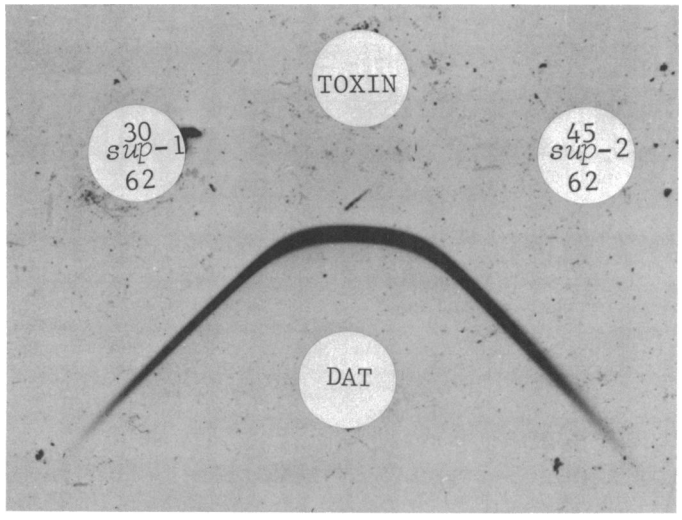

FIG. 4. *Radial immunodiffusion of diphtheria toxin and the 62,000-molecular-weight tox gene product isolated from culture supernatants of C7sup-1(β^{tox-30}) and C7sup-2(β^{tox-45}), (designated 30 sup-1-62 and 45 sup-2-62, respectively) against nonabsorbed diphtheria antitoxin (DAT).*

TABLE 2. *NAD:EF-2 ADPR-transferase activity and molecular weight of diphtheria tox gene products expressed by lysogenic strains of C. diphtheriae*

Corynephage	*C. diphtheriae* host[a]					
	C7*sup*$^+$(−)$^{tox^-}$		C7*sup-1*(−)$^{tox^-}$		C7*sup-2*(−)$^{tox^-}$	
	Enzyme activity	Mol wt (K)	Enzyme activity	Mol wt (K)	Enzyme activity	Mol wt (K)
β^{tox^+}	+	62	+	62	+	62
β^{tox-30}	+	30	+	62/30	+	30
β^{tox-45}	+	45	+	62/45	+	62/45
$\beta^{tox-111}$	+	47	+	62/47	+	62/47
$\beta^{tox-112}$	+	NDt	+	NDt	+	NDt
$\beta^{tox-113}$	+	NDt	+	NDt	+	NDt
$\beta^{tox-116}$	−	ND	−	ND	−	ND
$\beta^{tox-117}$	−	ND	−	ND	−	ND
β^{tox-1}	−	20	−	20	−	20
β^{tox-2}	+	26	+	26	+	26
β^{tox-3}	+	34	+	62/34	+	62/34
β^{tox-4}	−	ND	+	62	+	48
β^{tox-5}	−	ND	+	62	−	ND
β^{tox-6}	−	ND	+	62	+	62
β^{tox-7}	−	ND	−	ND	−	ND
γ^{tox^-}	−	ND	−	ND	−	ND

[a] NDt, Not determined; ND, not detectable.

into two groups; the first is suppressible, and the second is not.

There are many possible explanations for the inability to demonstrate suppression in all of the class II and III mutants examined. In general three classes of Sup$^-$ strains have been described: amber (2), ochre (3), and opal (21, 25). We have isolated only two different Sup$^-$ strains of *C. diphtheriae*. We are continuing to screen C7*sup*$^+$(−)$^{tox^-}$ clones that have survived mutagenesis for additional suppressor strains. The isolation of Sup$^-$ *C. diphtheriae* with different patterns of suppression should allow further characterization of class II and III mutants. At this time, we cannot rigorously rule out the possibility that phage β carries an unknown gene whose product is involved in the regulation of diphtheria *tox* gene expression. Mutations in this hypothetical gene might also be responsible for a CRM$^-$ phenotype. In addition, a CRM$^-$ mutant could have a lesion in a hypothetical promotor region for the *tox* gene. In the latter two cases, one would not necessarily expect the type of extragenic suppression described here to have any effect.

To date the genetic analysis of corynephage β has been made by the characterization of temperature-sensitive mutants (10, 17, 22) and by general recombination (12, 14). The availability of Sup$^-$ strains of C7*sup*$^+$(−)$^{tox^-}$ will provide another method for the study of β phage genetics and diphtheria *tox* gene regulation.

ACKNOWLEDGMENTS

This study was aided by grant PCM76-18924 from the National Science Foundation, and Public Health Service grant AI 12500 from the National Institute of Allergy and Infectious Diseases. J. R. Murphy is a recipient of Public Health Service Career Development Award K04 AI 100146 from the National Institute of Allergy and Infectious Diseases. P. Bacha has received a National Science Foundation Graduate Fellowship.

LITERATURE CITED

1. **Barksdale, W. L., and A. M. Pappenheimer, Jr.** 1956. Phage host relationships in nontoxigenic and toxigenic

diphtheria bacilli. J. Bacteriol. **67**:220–232.
2. **Benzer, S., and S. P. Champe.** 1962. A change from nonsense to sense in the genetic code. Proc. Natl. Acad. Sci. U. S. A. **48**:1114–1121.
3. **Brenner, S., and J. R. Beckwith.** 1965. Ochre mutants, a new class of suppressible nonsense mutants. J. Mol. Biol. **13**:629–637.
4. **Chung, D. W., and R. J. Collier.** 1977. Enzymatically active peptide from the adenosine diphosphate-ribosylating toxin of *Pseudomonas aeruginosa*. Infect. Immun. **16**:832–841.
5. **Cuatrecases, P., M. Wilchek, and C. B. Anfinsen.** 1968. Selective enzyme purification by affinity chromatography. Proc. Natl. Acad. Sci. U. S. A. **61**:636–643.
6. **Drake, J.** 1966. Mutagenic mechanisms. Annu. Rev. Genet. **3**:247–268.
7. **Gill, D. M., and L. L. Dinius.** 1971. Observations on the structure of diphtheria toxin. J. Biol. Chem. **246**:1485–1491.
8. **Gill, D. M., and A. M. Pappenheimer, Jr.** 1971. Structure activity relationships in diphtheria toxin. J. Biol. Chem. **246**:1492–1495.
9. **Groman, N. B., and M. Eaton.** 1955. Genetics factors in *Corynebacterium diphtheriae* conversion. J. Bacteriol. **70**:637–640.
10. **Groman, N. B., and W. Laird.** 1977. Heat-inducible mutants of corynebacteriophage. J. Virol. **23**:587–591.
11. **Holmes, R. K.** 1976. Characterization and genetic mapping of nontoxigenic (*tox*) mutants of corynebacteriophage beta. J. Virol. **19**:195–207.
12. **Holmes, R. K., and L. Barksdale.** 1969. Genetic Analysis of tox^+ and tox^- bacteriophage of *Corynebacterium diphtheriae*. J. Virol. **3**:586–598.
13. **Kanei, C., T. Uchida, and M. Yoneda.** 1977. Isolation from *Corynebacterium diphtheriae* C7(β) of bacterial mutants that produce toxin in medium with excess iron. Infect. Immun. **18**:203–209.
14. **Laird, W., and N. B. Groman.** 1976. Prophage map of converting corynebacteriophage beta. J. Virol. **19**:208–219.
15. **Laird, W., and N. B. Groman.** 1976. Isolation and characterization of *tox* mutants of corynebacteriophage beta. J. Virol. **19**:220–227.
16. **Lewin, B.** 1974. Bacterial genomes, p. 211–214. *In* Gene expression, vol. 1. John Wiley and Sons, New York.
17. **Matsuda, M., C. Danei, and M. Yoneda.** 1971. Temperature sensitive mutants of non-lysogenizing corynebacteriophage β^{ts}: their isolation, characterization, and relation to toxigenicity. Biken J. **19**:119–130.
18. **Murphy, J. R., A. M. Pappenheimer, Jr., and S. Tayart De Borms.** 1974. Synthesis of diphtheria *tox* gene products in *Escherichia coli* extracts. Proc. Natl. Acad. Sci. U. S. A. **71**:11–15.
19. **Murphy, J. R., J. Skiver, and G. McBride.** 1976. Isolation and partial characterization of a corynebacteriophage β, *tox* operator constitutive-like mutant lysogen of *Corynebacterium diphtheriae*. J. Virol. **18**:235–244.
20. **Pappenheimer, A. M., Jr., T. Uchida, and A. A. Harper.** 1972. An immunological study of the diphtheria toxin molecule. Immunochemistry **9**:891–906.
21. **Sambrook, J. F., D. P. Fan, and S. Brenner.** 1967. A strong suppressor specific for UGA. Nature (London) **214**:452–453.
22. **Singer, R. A.** 1973. Temperature sensitive mutants of toxigenic corynebacteriophage beta. I. Genetics. Virology **55**:347–356.
23. **Uchida, T., D. M. Gill, and A. M. Pappenheimer, Jr.** 1971. Mutation in the structural gene for diphtheria toxin carried by temperate phage β. Nature (London) New Biol. **233**:8–11.
24. **Uchida, T., A. M. Pappenheimer, Jr., and R. Greany.** 1973. Diphtheria toxin and related protein. I. Isolation and properties of mutant proteins serologically related to diphtheria toxin. J. Biol. Chem. **218**:3838–3844.
25. **Zipser, D.** 1967. UGA: a third class of suppressible polar mutants. J. Mol. Biol. **29**:441–445.