0095-1137/78/0007-0091$02.00/0
JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1978, p. 91-96

Vol. 7, No. 1
Printed in U.S.A.

Determination of *Corynebacterium diphtheriae* Toxigenicity by a Colorimetric Tissue Culture Assay

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Received for publication 12 August 1977

Chinese hamster ovary (CHO) cell cultures in microtiter wells are sensitive to growth inhibition and killing by picogram quantities of diphtheria toxin. In the absence of biologically active toxin, the CHO cell culture produces sufficient acidic metabolites to change the phenol red pH indicator from pink to yellow within 56 h. In the presence of 10 pg of toxin per well, growth inhibition can be observed microscopically within 24 h. Diphtheria toxin can be qualitatively assayed from culture supernatants of *Corynebacterium diphtheriae* or from β-phage agar plaque plugs. The colorimetric CHO cell assay method for determining toxigenicity allows for the large-scale screening of either diphtheria toxigenicity or antitoxin titration of sera.

The laboratory diagnosis of diphtheria is based upon the isolation of *Corynebacterium diphtheriae* and the demonstration of toxin production. Strains of *C. diphtheriae* that are lysogenic for corynebacteriophage β, or a family of closely related toxigenic corynephages, produce diphtheria toxin at maximal levels only during decline phase growth when iron is the limiting substrate (18). In general, the methods that have been used for the detection of diphtheria *tox* gene products have been based upon immunological reactivity (5, 9, 20) or toxigenicity (2, 10). In addition to toxigenicity testing in the laboratory, immunological reactivity has also been used as the criterion for selection of *C. diphtheriae* mutant lysogens that produce low levels of toxin (23), mutants that produce related nontoxic proteins that lack antigen determinants (8, 14), and both bacterial (11) and β-phage (17) mutant lysogens that produce toxin constitutively. Because immunological methods for toxin detection depend solely upon reactivity and not toxicity, they do not distinguish between nontoxigenic mutant strains that produce cross-reacting material and toxigenic strains that produce sufficient toxin to be detected. On the other hand, in vivo tests are extremely sensitive, but are costly and cumbersome. Laird and Groman (13) have developed a colony overlay test for toxigenicity that is based upon the sensitivity of HeLa cell monolayers to the toxin produced by *C. diphtheriae* that have grown on an agar overlay. After incubation, the overlay is removed, and the HeLa cell monolayer is stained. Intoxicated cells either fail to stain or stain poorly.

We have been interested in the regulation of diphtheria toxin production by *C. diphtheriae* and, in particular, the role of iron in the inhibition of toxin production. Our approach to this problem has been made, in part, by the isolation of mutant lysogens of *C. diphtheriae* that produce toxin even in the presence of inhibitory concentrations of iron (17). Progress in this area has been hampered by the lack of a convenient screening method for the isolation of Tox mutants. We report in this communication a rapid colorimetric tissue culture assay for determining the toxigenicity of *C. diphtheriae* isolates.

MATERIALS AND METHODS

Bacterial strains. The nonlysogenic *C. diphtheriae* $C7_s(-)^{tox-}$; the lysogenic, nontoxigenic *C. diphtheriae* $C7_s(\beta^{tox-30})$, $C7_s(\beta^{tox-45})$, $C7_s(\beta^{tox-197})$; and the lysogenic, toxigenic $C7(\beta^{tox+})$ and $C7_s(\beta^{tox+}_{cl1})$ have been described previously (17, 24). Cultures were maintained on chocolate agar plates. The nomenclature recommended by Holmes and Perlow (9) for mutant lysogenic strains of *C. diphtheriae* has been used.

Corynebacteriophage β. The corynebacteriophage β^{tox+}, β^{tox-30}, β^{tox-45}, and $\beta^{tox-197}$ were obtained from their lysogenic derivatives of C7 after ultraviolet irradiation induction essentially as described by Singer (21). The clear-plaque-forming mutants of corynephage β, β^{tox+}_c and β^{tox-45}_c, have been described previously (16).

Bacterial culture medium. Corynebacterial strains were grown in PT medium of the following composition: Casamino Acids (Difco), 10 g; 10% L-tryptophan, 10 ml; solution II (15), 2 ml; solution III (15), 1 ml; and 0.18% calcium pantothenate, 0.05 ml. The solution was made up to 1 liter, and the pH was adjusted to 7.2. Portions of 100 ml were autoclaved at 110°C for 15 min. Before use, 3 ml of sterile 50%

maltose–0.5% $CaCl_2 \cdot 2H_2O$ per 100 ml of medium was added aseptically.

Low-iron C-Y medium for the optimal production of diphtheria *tox* gene products has been described previously (17). Low-iron C-Y agar was made by the addition of 1 g of Noble agar (Difco) to 100 ml of C-Y broth. Portions of 100 ml were autoclaved at 110°C for 15 min and tempered to 50°C; 3 ml of 50% maltose–0.5% $CaCl_2 \cdot 2H_2O$ per 100 ml of medium was added aseptically, and the medium was dispensed into petri dishes. Low-iron top agar was prepared as described above, except for the concentration of Noble agar, which was added to 0.5%.

Base agar for phage titration contained 10 g of agar (Difco), 10 g of tryptose (Difco), 5 g of yeast extract, and 5 g of NaCl per liter (21). The pH was adjusted to 7.2, and the medium was autoclaved at 121°C for 15 min. Before dispensing, 3 ml of 50% maltose–0.5% $CaCl_2 \cdot 2H_2O$ per 100 ml of medium was added aseptically.

Phage dilutions were made in Neopeptone broth of the following composition (in grams per liter): Neopeptone (Difco), 10; yeast extract, 1; NaCl, 10; and KH_2PO_4, 2. The pH was adjusted to 7.2, and the broth was boiled and filtered through Whatman no. 40 paper and autoclaved in 100-ml portions for 15 min at 121°C (21).

Cell culture. The Chinese hamster ovary (CHO) cell line used in this study was derived from the K1 clone (12) and was kindly provided by R. L. Davidson, Department of Microbiology and Molecular Genetics, Harvard Medical School.

Cell culture medium and maintenance. CHO cells were grown in 25-cm^2 flasks (Corning, 25100) in Dulbecco-modified Eagle medium (DME) supplemented with 10% fetal calf serum, 2 mM glutamine, 1% nonessential amino acids, and penicillin and streptomycin to 50 IU and 50 μg/ml, respectively (Flow Laboratories, Inc., Rockville, Md.). Cells were grown at 37°C under 7% CO_2. Monolayers were harvested by rinsing with 5 ml of trypsin-ethylenediaminetetraacetic acid (Grand Island Biological Co., Grand Island, N.Y.), treating with 5 ml of trypsin-ethylenediaminetetraacetic acid at 37°C in 7% CO_2 for 10 min, mixing with an equal volume of fresh DME, and centrifuging at approximately 500 rpm in a clinical centrifuge for 5 min. The cell pellet was suspended in fresh DME to a final density of 1×10^5 cells per ml. Samples were transferred to new flasks or microtiter assay plates. CHO cells were transferred at intervals of 3 to 5 days, depending upon the acidity of the growth medium.

Toxigenicity tests. (i) CHO cell assay. All toxigenicity tests were performed in microtiter assay plates (Linbro, TS-FB-96). A 200-μl amount of CHO cell suspension (2×10^4 cells) was added to each well. To each assay well, purified diphtheria toxin, a β-phage agar plaque plug, or a portion of cell-free spent-culture medium of *C. diphtheriae* was added separately in the following manner. Purified diphtheria toxin (2,000 flocculating units per ml) was serially diluted in phosphate-buffered saline (PBS) containing 1 mg of bovine serum albumin (BSA) per ml, and 20 μl of each dilution was added aseptically to separate assay wells. β-Phage agar plaque plugs were cut from C-Y agar plates with sterile 50-μl disposable micropipettes and transferred singly to microtiter assay wells. β-Phage were grown on lawns of $C7_s(-)^{tox-}$ for 18 h at 34°C before picking. To test the toxigenicity of *C. diphtheriae* culture supernatants, strains were grown in 200-μl volumes of C-Y medium in microtiter plates under optimal conditions for toxin production. The bacteria were removed by centrifugation, and portions of 10 and/or 20 μl of each supernatant were added separately to assay wells.

(ii) Rabbit skin test. The rabbit intradermal test for diphtheria toxin was performed by the injection of 0.1 ml of a 1:100 dilution of cell-free culture supernatants from *C. diphtheriae*. Antitoxin controls were prepared in the following manner. Portions of 0.1 ml of culture supernatants were treated with 50 U of equine antitoxin (diphtheria antitoxin, lot SA-10; Massachusetts Antitoxin and Vaccine Laboratory, Jamaica Plain, Mass.), incubated at 37°C for 30 min, and diluted 1:50 with PBS, and 0.1 ml was injected intradermally into the backs of shaved New Zealand rabbits. Reactions were read at 24, 48, 72, and 96 h postinjection.

Quantitative immunoelectrohoresis of diphtheria toxin. Diphtheria toxin was measured by quantitative immunoelectrophoresis essentially as described by Axelsen et al. (1). One percent agarose was melted in 0.02 M barbital–0.013 M sodium acetate–0.001 M sodium azide (pH 8.6) (BAA) buffer in a boiling-water bath. Molten agar was tempered to 55°C before the addition of equine antitoxin to 0.5 U/ml, and 15 ml was then poured onto the surface of a glass plate (8.3 by 10.2 cm; Kodak projector slide cover glass, Eastman Kodak Co., Rochester, N.Y.). After the agar solidified, 2.5-mm-diameter holes were punched 10 mm from the lower edge of the plate. Ten-microliter samples were subjected to electrophoresis at 100 V for 4.5 h. BAA was used as the electrode buffer.

RESULTS

The sensitivity of CHO cell culture to diphtheria toxin is shown in Fig. 1. Serial twofold dilutions of purified toxin are made in PBS-BSA, and samples are transferred to the vertical rows 2 through 12 in the microtiter plate. The first vertical row serves as the antibody control. Dilutions of dialyzed antitoxin are made in PBS-BSA, and portions are transferred to the horizontal rows B through H in the microtiter plate. After incubation at 37°C under 7% CO_2, the presence of inhibitory concentrations of diphtheria toxin is observed colorimetrically. In those wells that do not contain inhibitory concentrations of toxin or in which the toxin has been neutralized by antitoxin, the CHO cells grow and produce sufficient metabolic end products to change the pH indicator from pink to yellow. In those wells that do contain inhibitory concentrations of toxin, the growth of the CHO cells is inhibited and the pH indicator remains pink. The microtiter plate shown in Fig. 1 was photographed through a green filter; the yellow

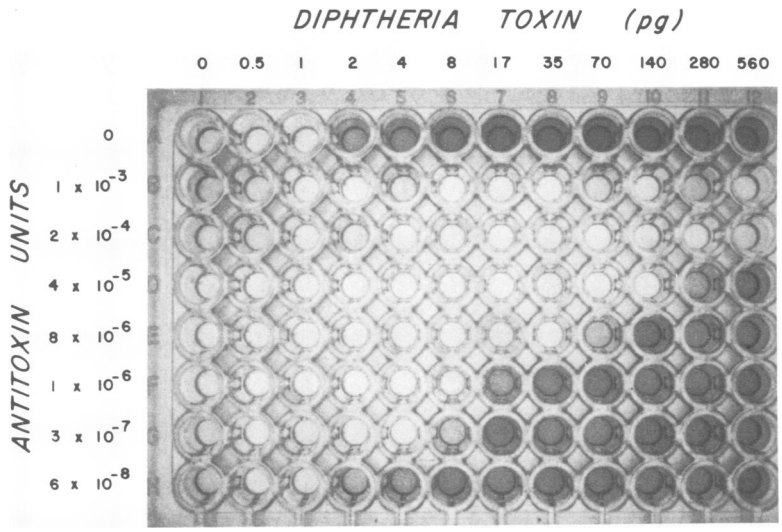

FIG. 1. *CHO cell assay of diphtheria toxin. Diphtheria toxin (vertical wells) and equine antitoxin (horizontal wells) were added to each well as indicated. CHO cells (2 × 10⁴ cells per well) in DME were dispensed, and the microtiter plate was incubated for 56 h at 37°C in an atmosphere containing 7% CO_2.*

wells appear colorless, whereas the pink wells appear gray. As shown in Fig. 1, the limit of sensitivity of the CHO cell assay for diphtheria toxin is approximately 2 pg after 56 h of incubation. Microtiter plates are read at the time that the control wells change from pink to yellow. In the presence of 2 pg of toxin per assay well, the CHO cells are only growth inhibited, and, therefore, a delay of 24 h in reading the assay plate results in a decrease in the sensitivity of the assay to 4 to 8 pg of toxin.

The inhibition of CHO cell growth by diphtheria toxin can be specifically neutralized by the prior addition of antitoxin to the microtiter wells. The maximum concentration of diphtheria toxin neutralized in each horizontal row is directly related to the concentration of antitoxin present. The ratio between the concentration of toxin neutralized and the concentration of antitoxin in each horizontal row is approximately constant and is in good agreement with the titer of the serum (1 U of equine antitoxin should neutralize approximately 2.5 μg of diphtheria toxin). The limit of sensitivity for the determination of antitoxin levels is on the order of 10^{-6} U/assay well. Because antitoxin is added in 10-μl volumes, the limit of sensitivity is 10^{-4} U/ml of sera.

Growth inhibition of CHO cells by diphtheria toxin can be observed microscopically within 24 h (Fig. 2). The generation time for this clone of CHO cells is approximately 8 h; therefore, in the absence of toxin, the CHO cell number will increase by about eightfold after 24 h of incubation. The addition of 10 pg of toxin to microtiter wells markedly inhibits the growth of the CHO cell culture within the first 24 h of incubation (Fig. 2). By 72 h, >90% of the cells appear rounded and have become detached from the culture dish. In the control wells, the CHO cells have grown out to a confluent monolayer within 48 h, and, by 56 h, the phenol red pH indicator will have changed from pink to yellow.

The addition of 10 μl of crude, cell-free culture supernatant from C7(β^{tox+}), grown under optimal conditions for toxin production, to microtiter assay wells also inhibits the growth of CHO cells. This growth inhibition is specifically neutralized by the addition of antitoxin to the assay medium. The minimal concentration of antitoxin necessary for the neutralization of 10 μl of C7(β^{tox+}) culture supernatant suggested the presence of 25 μg of diphtheria toxin per ml. This value is in excellent agreement with the concentration of 23 μg/ml determined by rocket immunoelectrophoresis. Cell-free culture supernatants of C7(β^{tox-30}), C7(β^{tox-45}), and C7($\beta^{tox-197}$), grown under optimal conditions for *tox* expression, and cell-free culture supernatants of the nonlysogenic, nontoxigenic C7 (−) are not inhibitory to the growth of CHO cells (Table 1).

The addition of 200 μM $FeSO_4$ to the growth medium of C7(β^{tox+}) has been shown to suppress the production of diphtheria toxin by >99.9% (17, 24). It was of interest, therefore, to assay cell-free culture supernatants of C7(β^{tox+}) grown in the presence of iron by the CHO cell method. Ten-microliter portions were not inhibitory to

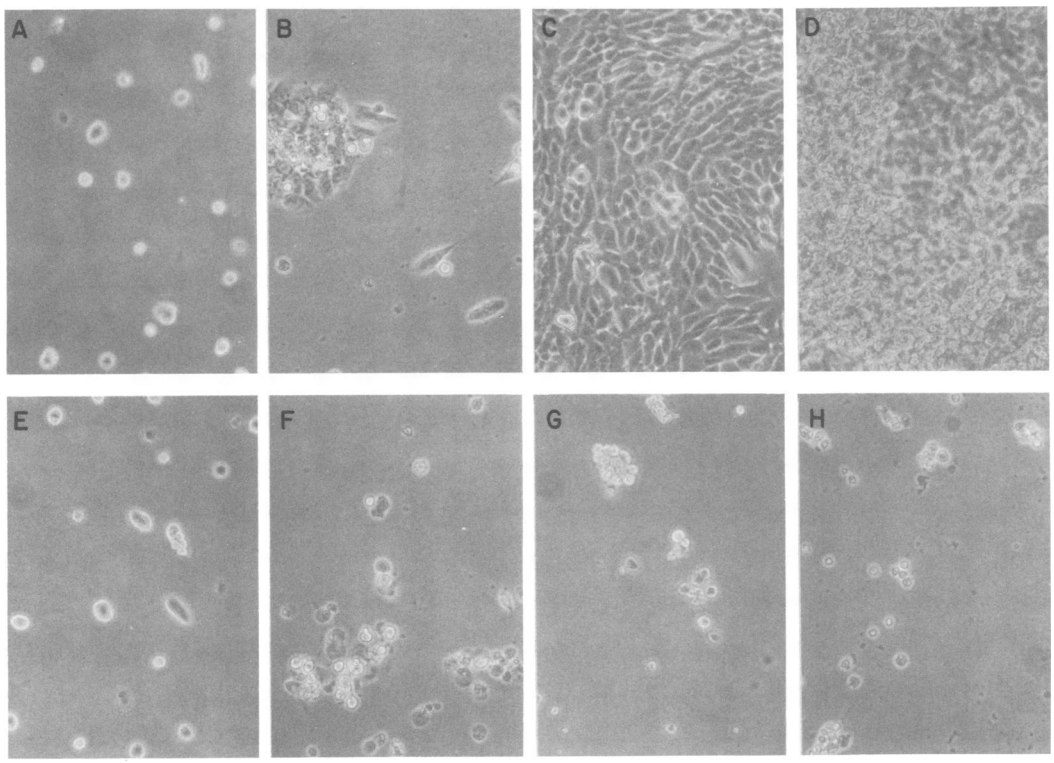

FIG. 2. *CHO cell growth in the absence (A through D) and presence (E through H) of 50 pg of diphtheria toxin per ml. (A) and (E) 0 h; (B) and (F) 24 h; (C) and (G) 48 h; (D) and (H) 72 h. Diphtheria toxin was added at 0 h.*

TABLE 1. *Comparison of the CHO cell assay and rabbit skin test determination of the toxigenicity of C. diphtheriae strains grown in C-Y broth*

C. diphtheriae strain	C-Y broth toxigenicity[a]			
	Deferrated		200 μM $FeSO_4$ supplemented	
	CHO	Rabbit[b]	CHO	Rabbit[b]
C7(−)	−	−	−	−
C7(β^{tox+})	+	+	−	−
C7(β^{tox-30})	−	−	−	−
C7(β^{tox-45})	−	−	−	−
C7($\beta^{tox-197}$)	−	−	−	−
C7(β^{tox+}_{cI1})	+	+	+	+
C7hm723 (β^{tox+})	+	+	+	+

[a] Toxigenicity tests were performed a minimum of 10 times.

[b] Culture supernatants were diluted 1:100 in PBS before intradermal injection.

the growth of CHO cells (Table 1).

Recently, two classes of mutants that produce high levels of diphtheria toxin in the presence of inhibitory concentrations of iron have been described (11, 17). The corynebacterial mutant C7hm723(β^{tox+}) (11) and the corynebacteriophage β mutant β^{tox-}_{cI1} (17) were grown in deferrated and 200 μM $FeSO_4$-supplemented C-Y medium. Ten-microliter portions of cell-free culture supernatants of both C7hm723(β^{tox+}) and C7(β^{tox+}_{cI1}) grown in the presence and absence of added iron were inhibitory to the growth of the CHO cells (Table 1).

Because the CHO cell assay is sensitive to low concentrations of diphtheria toxin, it was of interest to determine whether the toxin produced by toxigenic, lysogenic plaques could be detected on a lawn of sensitive C7(−). After 18 h of incubation at 34°C, individual plaques were punched out of C-Y agar plates with 50-μl disposable micropipettes and transferred to separate CHO cell assay wells in microtiter plates. Table 2 shows that C-Y agar plugs of β^{tox+} and β^{tox+}_{cI1} contain sufficient concentrations of diphtheria toxin to inhibit the growth of the CHO cell culture in the microtiter assay. A single β^{tox+} C-Y agar plaque plug was found to contain approximately 0.5 μg of diphtheria toxin. C-Y agar plaque plugs of the nontoxigenic β^{tox-30}, β^{tox-45}, and $\beta^{tox-197}$, as well as C-Y agar plugs from uninoculated plates, did not inhibit the growth

TABLE 2. *Toxigenicity of C-Y agar β plaque plugs in CHO cell culture*

C-Y agar plaque plug	C-Y agar[a]	
	Deferrated	200 μM $FeSO_4$ supplemented
Uninoculated	−	−
β^{tox+}	+	−
β^{tox-30}	−	−
β^{tox-45}	−	−
$\beta^{tox-197}$	−	−
β_{ctI}^{tox+}	+	+

[a] Toxigenicity tests were performed a minimum of 10 times.

of the CHO cells (Table 2). The addition of $FeSO_4$ to 200 μM in C-Y agar was sufficient to suppress the production of detectable levels of toxin in agar plaque plugs of β^{tox+} and β_c^{tox+} but not from the operator constitutive-like mutant β_{ctI}^{tox+} (Table 2). Because DME medium contains inhibitory concentrations of penicillin and streptomycin, *C. diphtheriae* from the surface of the C-Y agar plaque plug do not replicate and thereby interfere with the toxigenicity assay. Spontaneously released β-phage, on the other hand, can be recovered from the medium upon completion of the toxigenicity assay. Typically, 10^4 plaque-forming units per ml can be recovered from a single agar β-plaque plug by plating the CHO cell assay medium on a lawn of the sensitive $C7_s(-)$.

DISCUSSION

The isolation of corynebacteriophage β mutants that code for the production of nontoxigenic proteins that are serologically related to diphtheria toxin (22) and the in vitro synthesis of diphtheria *tox* products in S-30 extracts of *Escherichia coli* programmed with purified β-deoxyribonucleic acid (16) have conclusively demonstrated that the β-phage carries the structural gene for diphtheria toxin, *tox*. Diphtheria toxin is only produced at maximal levels when the host bacterium becomes starved for iron. The following two lines of evidence strongly indicate that the host bacterium plays a major role in the regulation of the β-phage *tox* gene: (i) the specific inhibition of *tox* expression in vitro by the addition of $C7_s(-)$ extracts to the *E. coli* protein-synthesizing system (16) and (ii) the isolation of corynebacterial mutants that constitutively produce diphtheria toxin (17). As stated above, studies on the regulation of diphtheria toxin production have been hampered, in part, by the lack of a rapid screening assay of the selection of both corynebacterial and corynebacteriophage β mutant lysogens in which the Tox phenotype is altered.

The CHO cell assay for diphtheria toxigenicity that we have described in this communication is sensitive, rapid, specific, and suitable for the large-scale toxigenicity testing of both corynebacterial and corynebacteriophage β potential mutants. We have screened as many as 1,500 potential Tox(−) mutants by either the agar plaque plug method or by the assay of culture supernatants of *C. diphtheriae* grown in microtiter plates. The sensitivity of the CHO microassay of diphtheria toxin routinely approaches that of the rabbit and guinea pig intradermal assay and the reversed passive hemagglutination assay recently described by Holmes and Perlow (9). Under optimal conditions, the CHO cell method as described can routinely detect between 10^7 and 10^8 molecules of diphtheria toxin. The level of sensitivity to toxin and its specific neutralization with antitoxin should allow for the development of a microassay for the determination of antitoxin levels in serum.

In general, microbial toxins have been shown to affect the physiology and/or morphology of sensitive eucaryotic cell lines. For example, diphtheria toxin in low concentrations has been shown to inhibit protein synthesis in a variety of cell lines (e.g., HeLa, BHK, CHO) derived from animals that are sensitive to the toxin. Significantly higher concentrations of toxin are required to inhibit protein synthesis in cell lines derived from resistant animals (e.g., mouse L-cells) (19). On the other hand, cholera toxin has been shown to stimulate adenylate cyclase in every cell line that has been examined so far (6). Stimulation of adenylate cyclase has been linked to morphological alterations (4, 7) and inhibition of cell growth (3). Ruch et al. (F. E. Ruch, J. R. Murphy, L. Graf, and M. Field, J. Infect. Dis., in press) have used the growth inhibition of S49 lymphosarcoma cells to screen potential nontoxigenic mutants of *Vibrio cholerae*. The S49 cell assay used for the detection of cholera enterotoxin activity is analogous to that described for diphtheria toxin in that toxigenicity is determined colorimetrically.

In the cases of diphtheria and cholera, the use of appropriate eucaryotic cell culture assays for toxigenicity has allowed the detection and subsequent isolation of Tox(−) mutants from large numbers of bacteria and, in the case of diphtheria, phage particles that have survived mutagenesis. The use of appropriate eucaryotic cell culture for the colorimetric determination of toxic activities from other procaryotes (e.g., PA toxin from *Pseudomonas aeruginosa*) will allow for the identification of toxigenic strains from nontoxigenic strains, the quantitative de-

termination of antitoxin levels in acute and convalescent sera, and the isolation of nontoxigenic mutants from large numbers of bacteria and/or phage that have survived mutagenesis.

ACKNOWLEDGMENTS

This research was aided by National Science Foundation grant BMS74-21102 and Public Health Service grant AI-12500-02 from the National Institute of Allergy and Infectious Diseases. J.R.M. is the recipient of Public Health Service career development award K04 AI-00146-01 from the National Institute of Allergy and Infectious Diseases.

We thank A. M. Pappenheimer, Jr., for his critical reading of the manucript.

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