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Author(s)	Hara, Tohru; Matsuda, Morihiro; Yoneda, Masahiko
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ISOLATION AND SOME PROPERTIES OF NONTOXIGENIC DERIVATIVES OF A STRAIN OF *CLOSTRIDIUM TETANI*

TOHRU HARA, MORIHIRO MATSUDA and MASAHIKO YONEDA[1]

Department of Tuberculosis Research I, Research Institute for Microbial Diseases, Osaka University, Yamada-kami, Suita, Osaka

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SUMMARY Nontoxigenic derivatives of a toxigenic strain of *Clostridium tetani* were isolated by treatment with acridine orange, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, rifampicin or ultraviolet light. The frequency of appearance of nontoxigenic derivatives on these treatments was 0.8 to 3.2 per cent. The nontoxigenic derivatives produced all the same extracellular antigenic and protein components as the toxigenic parent strain, except the toxin and materials cross-reacting with the toxin.

The nontoxigenic strains, like the toxigenic parent strain, were lyzed by treatment with mitomycin C. Bacteriophage was detected in the lysates of all the nontoxigenic derivatives produced with mitomycin C, and this bacteriophage was morphologically indistinguishable from that obtained from the toxigenic parent strain. The genetic factor controlling tetanus toxin production is discussed.

INTRODUCTION

Strains of *Clostridium tetani* produce a very potent toxin which mainly acts on the inhibitory synapses in the spinal cord (van Heyningen, 1971; Curtis, 1971). The toxin is synthesized within the young bacterial cells (Raynaud, 1951) as a single polypeptide chain with a molecular weight of approximately 150,000 daltons (Craven and Dawson, 1973; Matsuda and Yoneda, 1974). As the culture becomes older and the organisms autolyse, it is discharged into the culture medium in a nicked form consisting of two complementary polypeptide chains (Matsuda and Yoneda, 1974, 1975), showing increased toxicity (Seki et al., 1954; Lettl et al., 1966; Matsuda and Yoneda, 1974). Formation of the toxin is reported to require peptides containing histidine (Mueller and Miller, 1956) and to be almost completely inhibited by excess glutamate in the medium (Tsunashima et al., 1964). However, the mechanism of toxin production is unknown. The only reports on genetic aspects of toxin production are those by Prescott and Altenbern 1967a, 1967b) and by Nishida et al. (1969). Prescott and Altenbern (1967a) detected bacteriophage in mitomycin C induced lysates of toxigenic strains of *C. tetani*. Bacteriophages have been correlated with toxinogeny in several bacterial genera (Barksdale and Arden, 1974), but Prescott and Altenbern (1967a) showed that the mechanism of lysis induced by mitomycin C

1 Deceased on August 29, 1976.

was not related with the synthesis of tetanus toxin. Nishida et al. (1969) isolated nontoxigenic strains of *Clostridium* resembling *C. tetani* from soil and from a culture of a laboratory strain by heat selection and attempted to identify them as nontoxigenic *C. tetani* by H antigen agglutination. They suggested that there was an inverse relationship between the ability to sporulate and the degree of toxigenicity, but the exact relationship between the two properties is unknown. Thus very little is known about the genetic control of tetanus toxin production.

To study this problem, we isolated various nontoxigenic derivatives from an asporogenous, highly toxigenic strain of *C. tetani* by treatment with various agents and studied the antigenic and protein components which they produced in the culture medium and their lysogeny.

This paper reports the isolation and some properties of nontoxigenic derivatives of *C. tetani*. The nature of the genetic factor controlling tetanus toxin production is discussed on the basis of the findings.

MATERIALS AND METHODS

1. *Bacterial strains, media and cultures*

A substrain (Biken) of the asporogenous Harvard A47 strain of *Clostridium tetani* was used as the parent, toxigenic strain. Before use, three successive single-colony isolations of the strain were carried out on Zeissler's blood agar plates. The filtrates of a 4 day culture of the strain at 35 C in modified Latham medium (Matsuda and Yoneda, 1975) contained a concentration of 50 Lf units and $1–2\times10^6$ MLD per ml of toxin. The parent toxigenic strain and the nontoxigenic derivatives isolated from it were stored at 4 C in liver-liver broth after overnight growth at 35 C. Colonies isolated on Zeissler's blood agar plates from cultures which had been treated with various agents were each subcultured in semi-solid T.G.C. medium (Daigo Eiyokagaku Co.) and stored at 4 C, while their subcultures were each tested for toxigenicity. In early experiments, P-II medium (Kubota et al., 1960), which contains 10 g of whale heart extract (Nissui Seiyaku Co.), 20 g of polypeptone (Daigo Eiyokagaku Co.), 5 g of NaCl, 0.2 g of $MgSO_4\cdot7H_2O$, 0.16 g of $Na_2HPO_4\cdot12H_2O$, 0.1 g of KH_2PO_4, 10 g of glucose and 0.8 g of reduced iron per liter, pH 6.6–6.8, was used for tests on toxin production. Later, a modified Latham medium (Latham, Bent and Levine, 1962) was employed for toxin production; the Latham medium was modified by replacing 25 g of casein digest by 20 g of polypeptone and 10 g of whale heart extract and by adding 100 μg of folic acid per liter, pH 7.0 (Matsuda and Yoneda, 1975). For analysis of the protein components in the culture filtrates of the nontoxigenic derivatives, a protein-free, modified Latham medium was used, in which the 10 g of whale heart extract was replaced with the materials that dialyzed through Visking tube into distilled water from 15 g of whale heart extract, per liter of the medium. TYC broth contains 30 g of trypticase (BBL), 20 g of yeast extract (Difco), 1 g of cysteine hydrochloride (Wako Pure Chemical Industries, Ltd.) and 10 g of glucose per liter, pH 7.0. All cultures were incubated at 35 C unless otherwise stated.

2. *Treatment of the toxigenic cultures with various agents*

1) Acridine orange treatment

A sample of 0.1 ml of an overnight culture in liver-liver broth of the toxigenic parent strain was added to 15 ml of fresh liver-liver broth containing 25 μg of acridine orange (AO) per ml and cultured overnight.

2) *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine treatment

N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) was added at a concentration of 10 μg per ml to an actively growing culture (OD at 590 nm, 0.3) of the toxigenic parent strain in TYC broth and incubated at 35 C for 30 min. Then the culture was centrifuged at 4 C at 10,000 rpm for 15 min and the pellet was resuspended in TYC broth and small aliquots of the treated cell suspension were inoculated into tubes containing 6 ml of liver-liver broth and incubated further overnight.

3) Rifampicin treatment

A sample of 0.1 ml of an actively growing culture (OD at 590 nm, 0.3) of the toxigenic parent strain in TYC broth was inoculated into modified Latham medium (6 ml) containing 0.005 μg of rifampicin (RP) per ml and cultured overnight.

4) Ultraviolet light irradiation

For irradiation, a culture of the toxigenic parent strain in the logarithmic phase of growth (OD at 590 nm, 0.1) in TYC broth was diluted 10-fold with sterile 0.1 M potassium-sodium phosphate buffer, pH 7.5, and irradiated in 3 ml volumes in Petri dishes for 15 sec periods at a distance of 40 cm (Hitachi germicidal lamp 15 w) with gentle shaking.

3. *Selection of nontoxigenic derivatives*

The culture of the toxigenic parent strain, treated with various agents as described above, were each streaked onto Zeissler's blood agar plates. The colonies obtained by incubating the plates in an anaerobic jar for 48 hr were each subcultured into T.G.C. medium.

Volumes of 0.1 ml of overnight cultures were further inoculated into the modified Latham medium and incubated for 4 days to examine toxin production. The lysed culture was mixed with one-hundredth volume of 1% merthiolate and tested in vitro for the presence of antitoxin precipitable material in Ouchterlony's immunodiffusion plates against absorbed horse antitoxin serum (50 U/ml), using toxin (50 Lf/ml)-antitoxin (50 U/ml) precipitation bands as references. The culture filtrates of " nontoxigenic " derivatives, that is cultures which showed no precipitation with antitoxin, even when concentrated 10-fold, were tested further by injecting 0.5 ml samples subcutaneously into OF1 mice to see if they induced any symptoms of tetanus.

4. *Preparation of concentrated culture filtrates*

Culture filtrates of the organisms grown in modified Latham medium for 4 days were concentrated to approximately one-tenth of their original volume, either with Carbowax 20M (Wako Pure Chemical Industries, Ltd.) and then dialysis at 4 C against 0.1 M potassium-sodium phosphate buffer, pH 7.5 or in a minicon A25 macrosolute concentrator (Amicon Corp., Mass.).

5. *Toxin*

Tetanus toxin was prepared from bacterial extracts and purified as described previously (Matsuda and Yoneda, 1974).

6. *Antisera*

1) Horse antitoxin serum

Horse antitoxin serum prepared by immunizing a horse with crude toxoid preparation (crude horse antitoxin serum) and the absorbed antitoxin serum prepared by removal of the nonspecific antibodies from this serum by the method of Tsunashima et al. (1964) were gifts from Kanonji Institute, The Research Foundation for Microbial Diseases of Osaka University, Kanonji, Kagawa.

2) Rabbit antisera

Rabbit anti-fragment α and anti-fragment β sera were prepared as reported previously (Matsuda and Yoneda, 1976).

7. *Polyacrylamide gel electrophoresis*

Conventional polyacrylamide gel electrophoresis using 7.5% gel was carried out with 10 mM tris-(hydroxymethyl)aminomethane (Tris)-77 mM glycine buffer, pH 8.6, as the electrode buffer according to the method originally described by Davis (1964). Gels were stained with Amido black 10B (E. Merck, Darmstadt) and destained by electrophoresis in 7% acetic acid.

8. *Crossed immunoelectrophoresis*

Crossed immunoelectrophoresis on a micro-scale was carried out on 5.5×5.5 cm glass plates by the method of Laurell (1965). The antigens (6 μliters of concentrated culture filtrate, 10 mg/ml) were initially subjected to electrophoresis in an agarose gel (1%) containing 50 mM Tris–0.6 M glycine buffer, pH 8.5, containing 1 mM EDTA, applying 10 v/cm for 90 min at 4 C (first dimention electrophoresis). Then the electric field was turned through 90°, and electrophoresis was continued into agarose gel (1%) containing antiserum (concentration of antiserum containing antitoxin 5 U/ml of gel), applying a current of 2 v/cm for 20 hr at room temperature (second dimension electrophoresis). Gels were stained using Coomassie brilliant blue and destained by diffusion in ethanol-acetic acid-water (4.5 : 1 : 4.5, by volume).

9. *Immunodiffusion test*

The double diffusion precipitation method originally described by Ouchterlony (1953) was employed using 1% agarose containing 0.5 M glycine in buffered saline.

10. *Protein determination*

Protein was estimated by the method of Lowry et al. (1951).

11. *Mitomycin C induction*

Kinetic studies on induction of lysis with mito-

mycin C were performed in tubes (1.2×10.4 cm) from Bausch & Lomb-Shimazu Spectronic 20, containing 5 ml of TYC broth. Tubes were inoculated with an overnight culture in liver-liver broth to give an optical density (OD) of 0.01, and incubated until the OD attained a value of 0.1. Then various concentrations of mitomycin C (1, 2.5, 5, 7.5 and 10 μg/ml) were added and the OD was measured in a Bausch & Lomb-Shimazu Spectronic 20 spectrophotometer at 590 nm.

12. *Preparation of bacteriophage for electron microscopy*

After complete lysis of the cells with mitomycin C (10 μg/ml), the lysate was clarified by centrifugation at 4 C at 8,000×*g* for 20 min. The resulting supernatant fluid was centrifuged at 4 C at 54,500×*g* for 100 min in a Type 30 roter of a Beckman model 65 B ultracentrifuge. The pellet from 50 ml of clarified lysate was resuspended in 0.2 ml of 0.1 M ammonium acetate solution, pH 6.8, and examined in an electron microscope (Hitachi HU-12A) by the negative staining technique with 2% phosphotungstic acid, adjusted to pH 6.8 with KOH. In some cases, phage was further purified using a stepwise CsCl density gradient (densities: 1.3, 1.5 and 1.7 g/cm^3) as described by Mathews (1960).

13. *Chemicals*

Acridine orange was purchased from E. Merck AG, Darmstadt, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine from Aldrich Chemical Co., rifampicin from Daiichi Seiyaku Co., and mitomycin C from Kyowa Hakko-kogyo Co.

RESULTS

1. *Isolation of nontoxigenic derivatives from the toxigenic strain*

Nontoxigenic colonies were obtained by treating the toxigenic strain with various agents (Table 1). Table 1 shows that the frequency of incidence of the nontoxigenic derivatives on treatment was 0.8 to 3.2 per cent, but that no nontoxigenic colonies were obtained without treatment. The morphological features, gram-stainability and doubling times in various media of the nontoxigenic derivatives and the toxigenic parent strain were similar. Reversion from nontoxigenicity to toxigenicity did not occur during repeated subcultures of the nontoxigenic derivatives.

2. *Antigenic properties of culture filtrates of the nontoxigenic derivatives*

Figure 1 shows the immunodiffusion patterns of concentrated culture filtrates of a nontoxigenic derivative and the toxigenic parent strain against crude horse antitoxin serum (Fig. 1A) and of rabbit antisera against fragments of tetanus toxin (Fig. 1B). Against crude horse antitoxin serum, the concentrated culture filtrates of the nontoxigenic derivative formed multiple precipitation bands which all fused with those between the antiserum and concentrated culture filtrates of the toxigenic parent strain, except for a single precipitation band of toxin-antitoxin (Fig. 1A). Figure 1B shows the absence of either fragment α or fragment β of tetanus toxin (Matsuda and

TABLE 1. *Loss of toxigenicity of C. tetani on treatment with various agents*

Treatment with	Total no. of colonies tested	Nontoxigenic derivatives	
		No. of colonies	Per cent
Acridine orange	519	4	0.8
Rifampicin	150	2	1.3
N-methyl-*N'*-nitro-*N*-nitrosoguanidine	690	6	0.9
Ultraviolet light	126	4	3.2
Untreated	562	0	0

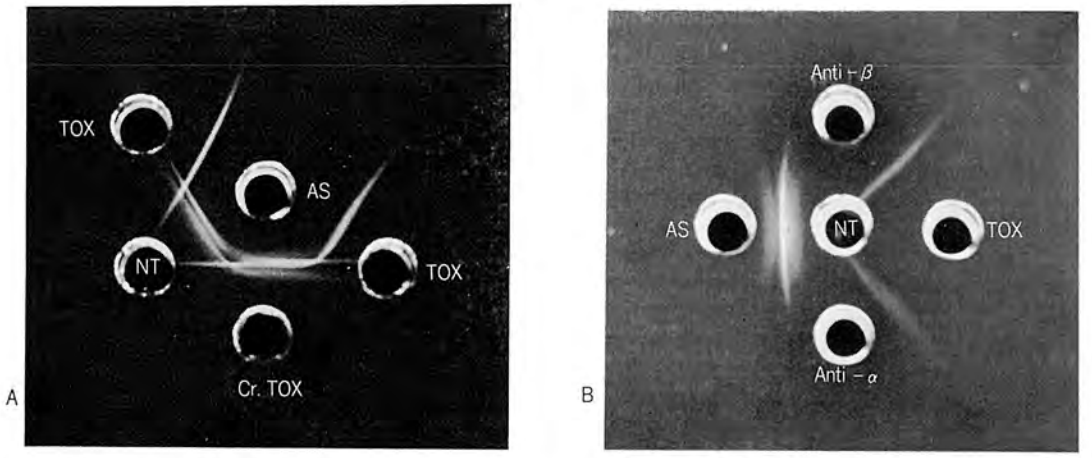


FIGURE 1 Immunodiffusion patterns of the concentrated culture filtrates of a nontoxigenic derivative and the toxigenic parent strain against crude horse antitoxin serum (A) and against rabbit anti-fragment α and anti-fragment β sera (B). Cr.TOX, culture filtrate of toxigenic strain (11 mg protein/ml); NT, culture filtrate of a nontoxigenic derivative, AO 174 (10 mg protein/ml); TOX, purified tetanus toxin (1 mg/ml for (A) and 500 μg/ml for (B)). AS, crude horse antitoxin serum (400 U/ml); Anti-α, anti-fragment α (20 toxin neutralizing units/ml); Anti-β, anti-fragment β (100 toxin neutralizing units/ml).

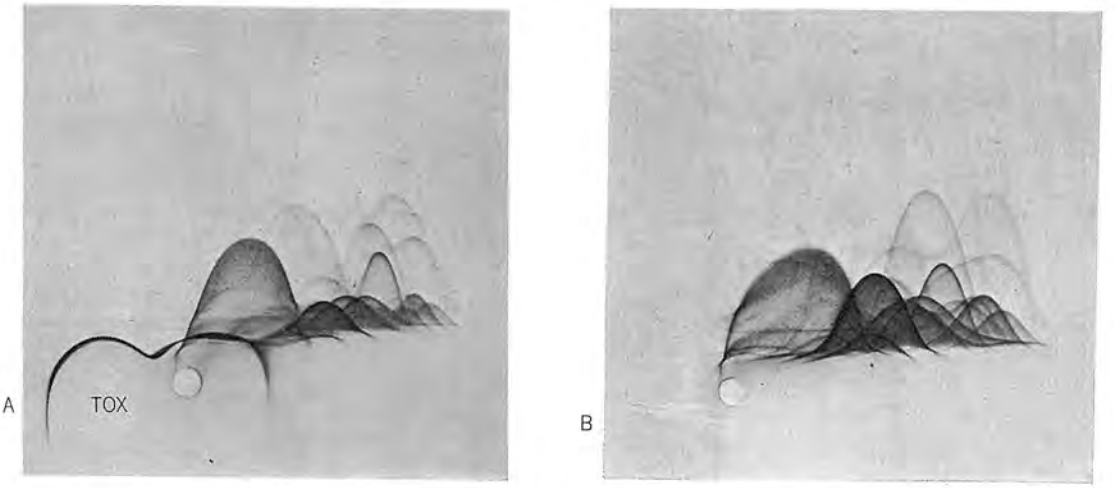


FIGURE 2 Crossed immunoelectrophoresis of the culture filtrates of the toxigenic parent strain (A) and a nontoxigenic derivative, AO 174 (B). Samples of 6 μliters of antigen (11 mg and 10 mg protein per ml for (A) and (B), respectively) were applied. First dimension electrophoresis, anode to the right. Second dimension electrophoresis, anode at the top. Just before electrophoresis in the second dimension, 6 μliters of purified toxin (1 mg/ml) was added to the TOX well as a reference.

Yoneda, 1975, 1977) in the concentrated culture filtrates of the nontoxigenic derivative. Similar results were obtained with culture filtrates of all the nontoxigenic derivatives. All the "nontoxigenic" derivatives selected by in vitro immunodiffusion tests using 10-fold concentrated culture filtrates were also nontoxigenic in an in vivo toxicity test, in which 0.5 ml volumes of the concentrated culture filtrates were injected subcutaneously into mice.

The antigenic components of the nontoxigenic derivatives were also compared with those of the toxigenic parent strain by antigen-antibody crossed electrophoresis using crude horse antitoxin serum. As seen in Fig. 2, with this sensitive test, more than twenty-five *C. tetani* antigens were detected in both preparations (Fig. 2A, B): the numbers of antigens and the patterns of crossed immunoelectrophoresis were similar except for one precipitation arc, which was identified as that of toxin-antitoxin by the purified toxin in the reference well (Fig. 2A). The toxin-antitoxin arc was detected only in the culture filtrate of the toxigenic parent strain (Fig. 2A). These results indicate that the culture filtrates of the nontoxigenic derivatives and the toxigenic parent strain contain similar quantities of all the antigenic components of *C. tetani* detectable with crude horse antitoxin serum, except tetanus toxin.

3. *Polyacrylamide gel electrophoresis of the culture filtrates of the nontoxigenic derivatives*

Figure 3 shows the profiles on polyacrylamide gel electrophoresis of the protein components in the culture filtrates of representative nontoxigenic derivatives, and the toxigenic parent strain. All the filtrates gave numerous protein bands (Fig. 3B, C, D, E), but the main protein band (Fig. 3B) of the toxigenic parent strain, which is that of toxin (Fig. 3A), was not formed by any of the nontoxigenic derivatives (Fig. 3C, D, E). The numbers and relative quantities of protein components other than toxin were similar in filtrates of the toxigenic parent strain and the nontoxigenic derivatives.

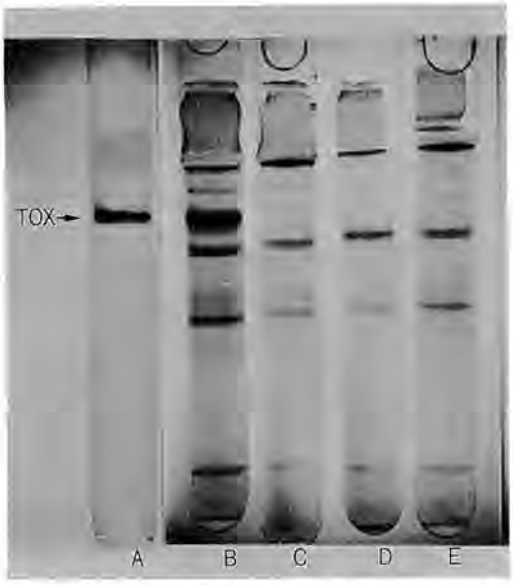


FIGURE 3 Polyacrylamide gel electrophoresis of the culture filtrates of the toxigenic parent strain (B) and nontoxigenic derivatives (C, D, E). Samples of 10 μliters of purified tetanus toxin (1 mg/ml) for (A) and of 50 μliters (ca. 1.6 mg/ml) of the culture filtrates of the toxigenic parent strain (B), and nontoxigenic derivatives, RP 81 (C), AO 5 (D) and NG 1 (E) were applied. Electrophoresis was performed for 3 hr at a constant current of 1.5 ma/gel. Migration was from top to bottom.

4. *Induction of nontoxigenic derivatives with mitomycin C*

To examine whether the nontoxigenic derivatives obtained were cured of phage, we tested filtrates of mitomycin C induced lysates of the toxigenic parent strain for formation of spots on lawns of each nontoxigenic derivative. No spots were found. In further studies on the lysogeny of the nontoxigenic derivatives, we compared the effects of mitomycin C on cultures of the nontoxigenic derivatives and the toxigenic parent strain. The nontoxigenic derivatives and the toxigenic parent strain had similar doubling times (about

90 min) in TYC broth and showed similar degrees of sensitivity to mitomycin C. Moreover, the nontoxigenic derivatives were lysed by mitomycin C in a similar way to the toxigenic parent strain. Representative results of induction experiments are shown in Fig. 4. Lysis began at about 4 hr and was complete 6–7 hr after addition of mitomycin C. A concentration of 5–10 μg/ml of mitomycin C caused maximal lysis of both nontoxigenic derivatives and the toxigenic parent strain.

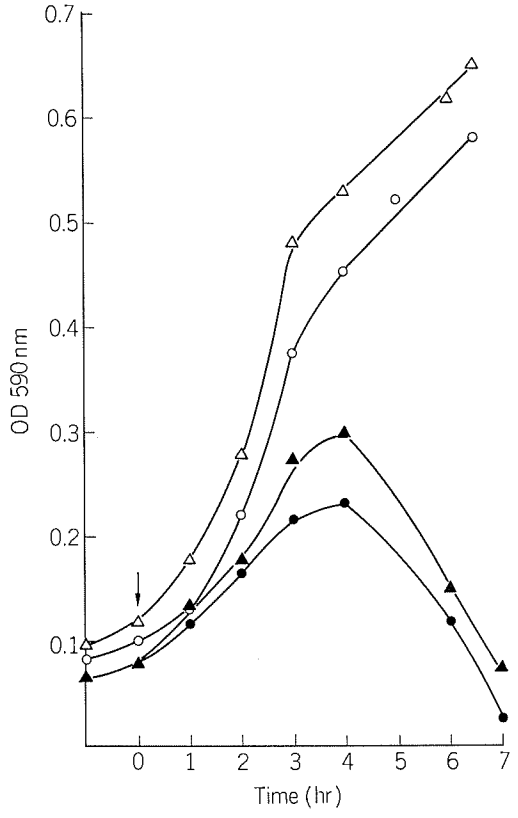


FIGURE 4 Changes of the optical density of the cultures of the toxigenic parent strain (●) and a nontoxigenic derivative, AO 58 (▲) treated with mitomycin C. Mitomycin C (final concentration 10 μg/ml) was added at time zero (indicated by arrows). ○ (Toxigenic parent strain) and △ (nontoxigenic derivative, AO 58), mitomycin C not added.

5. *Presence of bacteriophage in mitomycin C induced lysates of the nontoxigenic derivatives*

The pellets prepared from mitomycin C induced lysates of representative nontoxigenic derivatives obtained by treatment with each agent were examined for the presence of bacteriophage. Figure 5 shows electron micrographs of bacteriophage of the toxigenic parent strain (Fig. 5A) and that of a nontoxigenic derivative (Fig. 5B). The bacteriophage of the nontoxigenic derivative had the typical morphology of a temperate phage mainly with an electron-dence hexagonal head, 55–60 nm in diameter, and a tail, 100–110 nm long and 5–8 nm in diameter, without a sheath (Fig. 5B). Bacteriophage indistinguishable in size and morphology from that of the toxigenic parent strain (Fig. 5A) was observed in lysates of all the nontoxigenic derivatives examined.

DISCUSSION

The present results show that after treatment of cultures of *C. tetani* with various agents, nontoxigenic derivatives could be isolated with a far higher frequency than that expected for an occurrence due to mutations in bacterial chromosomes. Thus the derivatives could be isolated without using any special selection method. The nontoxigenicity of the derivatives seemed to be very stable, because no toxigenic revertants were recovered from the nontoxigenic cultures on repeated subcultures over a period of years. The nontoxigenic derivatives did not produce toxin, unlike the parent strain which produces as much as 5–10% of the weight of the bacterial cells, without any inhibition of their growth. The toxin does not appear to be essential to the life of the microorganism.

Pathogenic clostridia are mainly identified on the basis of the characteristic toxins which they produce. Thus it is difficult to identify nontoxigenic clostridia resembling toxigenic clostridia (Takahashi et al., 1967; Nishida et al., 1969). However, in this study, we iden-

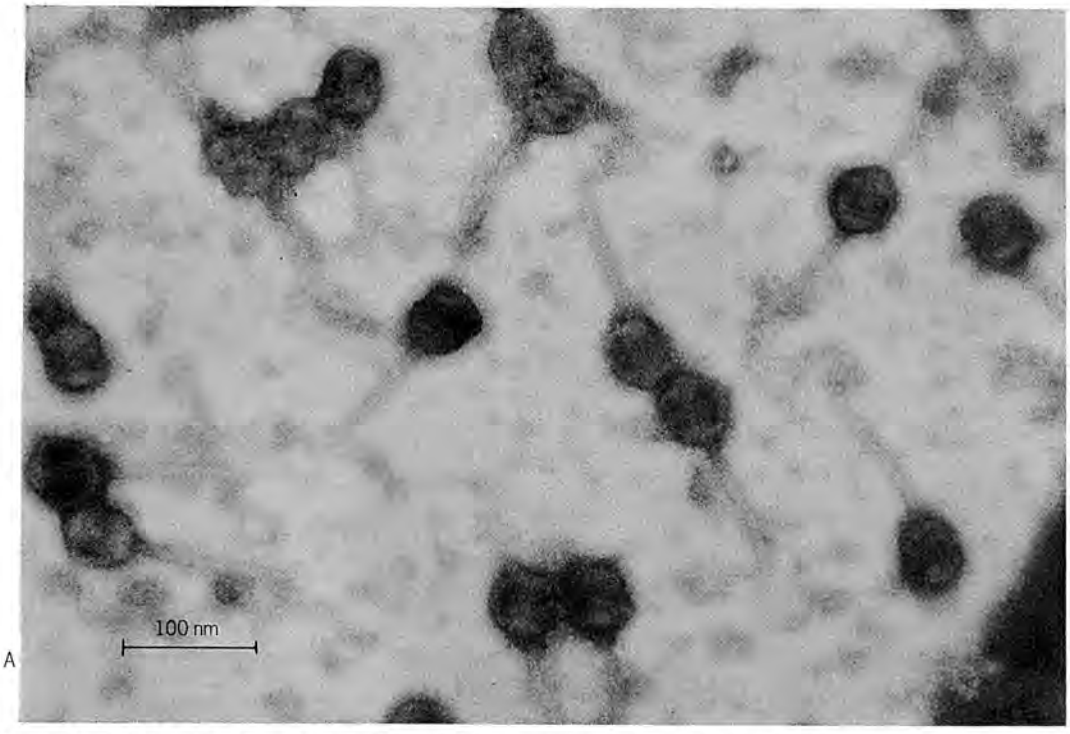
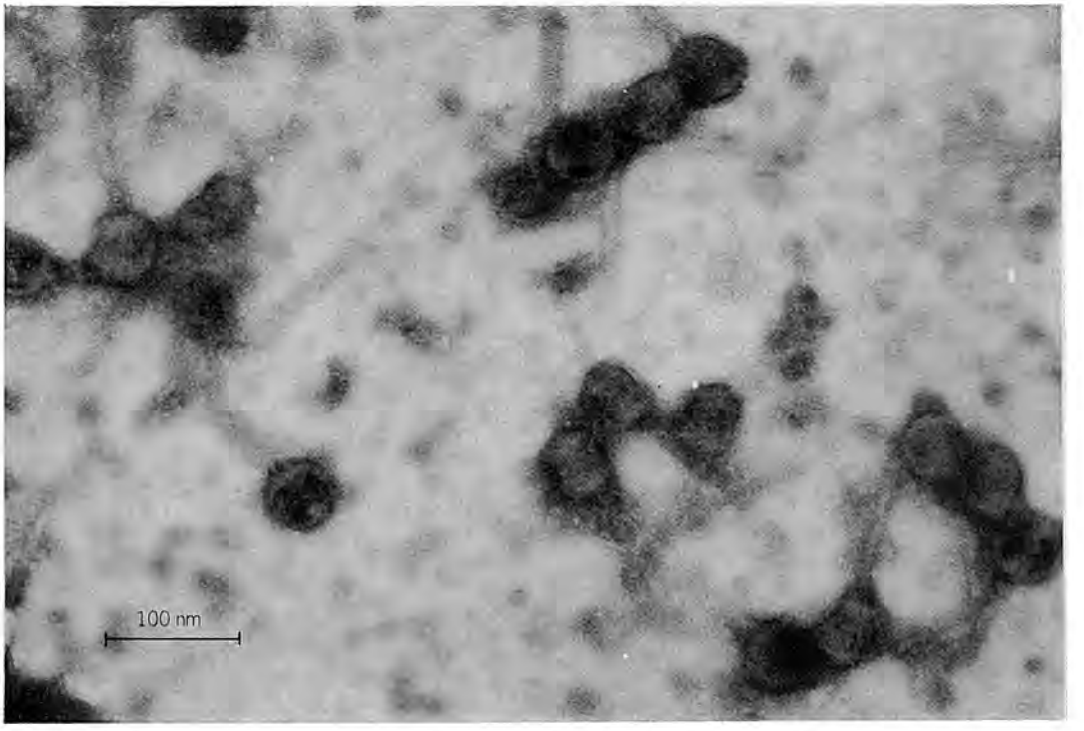


FIGURE 5 ▲▶Bacteriophage from mitomycin C induced lysates of *C. tetani*. A, toxigenic parent strain, Harvard A47 and B, a nontoxigenic derivative, AO 174. Scale : 100 nm.

tified nontoxigenic derivatives of *C. tetani*, not only on the basis of the general properties of the species, but also immunochemically using antiserum against crude culture filtrates of the parent strain, which contain multiple antigens of the *C. tetani* parent strain: analysis showed that loss of the ability to produce toxin appeared to be limited to a single antigen, toxin protein, in all the nontoxigenic derivatives isolated. The nontoxigenic derivatives did not produce either whole toxin molecules or fragments of the toxin. These results were supported by absorption experiments with *C.* tetani antigens free from toxin, obtained from a nontoxigenic derivative, as reported in the accompanying paper (Hara, Matsuda and Yoneda, 1977).

It has been suggested that the production of toxin by *C. tetani* might be governed by the presence of a specific converting bacteriophage, in a system analogous to that for the production of toxin by *C. botulinum* type C and D (Inoue and Iida, 1970; Eklund et al., 1971; Eklund, Poysky and Reed, 1972). In electron microscopic studies, Prescott and Altenbern (1967b) obserbed *C. tetani* phages of a different morphology in pellets obtained from mitomycin C induced lysates of toxigenic strains, 10779 and 453, of *C. tetani*. In this work we found only a single kind of phage in lysates of the toxigenic parent strain. This is a substrain (Biken) of the Harvard A47, and it has a bacteriophage morphologically similar to that reported for strain 10779, which has an ancestor in common with our strain. In contrast two different phages have been isolated from *C. botulinum* strain 468 (Eklund et al., 1971). Consistent with our failure to detect



B

plaques on lawns of the nontoxigenic derivatives using a mitomycin C lysate of the toxigenic parent strain, electron microscopic observation showed that none of the nontoxigenic derivatives were " cured " of their " prophage ". So far, no suitable indicator strain that can detect the plaque forming activity of the phage has been found for use in further precise studies. However, the phage of the nontoxigenic derivatives was as functionally inducible and morphologically intact as that of the parent strain. Thus it is tentatively concluded that the mere presence of bacteriophage is not directly responsible for tetanus toxin production, in sharp contrast to the cases of *C. botulinum*, where toxigenic cultures could be simultaneously cured of their prophage and toxigenicity. However, it must be pointed out that recently Oguma, Iida and Inoue (1975) obtained a nonconverting phage, c-n71 from a nontoxigenic derivative C-*N*71 of *C. botulinum* type C, which is indistinguishable in morphology, lytic activity, antigenic specificity or immunity from the converting phage, although it is not yet certain whether c-n71 completely lacks genetic information for toxin production. A similar situation seems to exist in the case of " lysogenic ", nontoxigenic derivatives of *C. tetani*. However, there are some differences between these cases: nontoxigenic derivative C-*N*71, lysogenic for a nonconverting phage c-n71 is rather an exceptional case in *C. botulinum*, which has so far been isolated only from the NG treated cultures, whereas all the nontoxigenic derivatives of *C. tetani*, were not " cured " of phage regardless of the kind of agents (AO, RP, NG or UV) used for the isolation. Before any definite conclusion can be made on the role of phage in tetanus toxin production, *C. tetani* which has been cured of its prophage but still remains toxigenic must be isolated.

Various chemicals, including acridine orange and rifampicin, have been used to remove ex-

trachromosomal elements from bacteria. The high rate of loss of toxigenicity, the stability of the loss, the restriction of the loss to the single component, toxin, and the fact that toxin is not essential for bacteria, all seems to indicate that the genetic factor controlling tetanus toxin production is an extrachromosomal element, plasmid, not directly related to the phage which *C. tetani* carries. It is possible that the plasmid may be transduced by the bacteriophage. If the genetic factor controlling tetanus toxin production were found to be a plasmid, this finding would provide an important clue to the confusing problem of classification of nontoxigenic clostridia resembling *C. tetani* (Sanada, 1964; Nishida et al., 1969). Repeated attempts to show direct transmission of toxigenicity from the toxigenic parent strain to the nontoxigenic derivative, recipient, by mixed culture, have so far been unsuccessful. Studies on the isolation of closed circular DNA from the toxigenic strain and search for a suitable indicator bacteria for the phage of *C. tetani* are in progress in our laboratory.

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