INFECTION AND IMMUNITY, Oct. 1977, p. 203-209
Vol. 18, No. 1
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Isolation from *Corynebacterium diphtheriae* C7(β) of Bacterial Mutants That Produce Toxin in Medium with Excess Iron

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Received for publication 6 April 1977

Five mutants that produce toxin in medium with excess iron were isolated from strain C7(β). The iron content of bacteria grown on this medium was considerably higher than that of C7(β) cells grown in medium containing the minimum amount of iron needed to inhibit toxin production. When the nonlysogenic, nontoxinogenic strain C7(−) was lysogenized with phages from each of the mutants, toxin production by all of the resulting lysogens, like that by parent strain C7(β), ceased upon iron addition. When the mutants were superinfected with β45 phage, both toxin and CRM45 were produced in medium with excess iron. One of the mutant strains lost its prophage as a result of treatment with ultraviolet light. When the cured strain was lysogenized with phage carrying a mutation in the *tox* structural gene, the lysogen produced the mutant protein at the maximum rate in medium with excess iron. These findings show that the mutant strains are not phage mutants, but are bacterial host mutants, and that a host factor(s) is involved in the inhibition of toxin production by iron.

Diphtheria toxin is synthesized de novo (24) and released extracellularly by *Corynebacterium diphtheriae* strains such as C7(−), after either lysogenization (3, 7, 10) or lytic infection (15) with β phages carrying the toxin structural gene (28, 29).

Toxin production is inhibited after iron addition to *C. diphtheriae* cultures (14, 23, 25), whereas phage multiplication is not affected (11). The *tox* gene can be expressed in the absence of phage multiplication and is, therefore, under separate control from systems regulating the expression of other phage genes (16, 18, 34).

Although much attention has been paid to the inhibition of toxin production by iron (2, 6, 27), the mechanism itself is still unknown. Recently, Murphy et al. (20) have reported that diphtheria toxin or CRM45 was synthesized in an in vitro system extracted from *Escherichia coli* supplemented with deoxyribonucleic acid from β or β45 phage. Their preliminary experiments showed that both toxinogenic and nontoxinogenic strains of diphtheria bacilli contain a factor that blocks expression of the *tox* gene.

During, the course of studies on the mechanism of inhibition of toxin production by iron, we isolated mutants that produce toxin at the normal rate in medium containing excess iron. This paper reports the isolation and characterization of several such mutants. Our present study suggests that a bacterial host factor(s) is involved in the mechanism by which iron inhibits toxin production by C7(β) cells.

(Part of this work was presented at the 47th Annual Meeting of the Japanese Society for Bacteriology, Kyoto, Japan, 2 to 4 April 1974 [C. Kanei, T. Uchida, and M. Yoneda. Jpn. J. Bacteriol. Soc. **29**:193, 1974].)

MATERIALS AND METHODS

Bacterial strains. Mutants were isolated from *C. diphtheriae* C7(β)$^{tox^+}$. Strain C7(−)$^{tox^-}$ was used as indicator strain for β phage.

Phage strains. Phages β, β45, β197 (30), and β-NG2 (17) were prepared from ultraviolet light-induced C7(β), C7(β45), C7(β197), and C7(β-NG2). Phages related to each bacterial mutant were prepared from ultraviolet light-induced mutant strains. Phage β45c, which was used for superinfection, was a clear-plaque mutant of phage-β45 carrying the structural gene of toxin mutant protein, cross-reacting material (CRM45; molecular weight, 45,000) (28).

Media. PGT medium (2) and modified Linggood-Fenton medium (13) were used for toxin production and growth. CST-6 medium containing succinate as the sole energy source was used for toxin production under growth-limiting conditions (12). Antitoxin-agar medium contained (per liter): tryptose (Difco certified), 10 g; agar (Difco certified), 10 g; NaCl, 5 g; 10% $CaCl_2$, 2 ml; Mueller solution II (19), 4 ml; and 10% cystine, 2 ml. It was adjusted to pH 7.2 with NaOH. Before the agar medium was poured into petri dishes, final concentrations (per milliliter) of 1.5 flocculating units of horse antitoxin serum (supplied from the Vaccine

Production Plant of our Institute) and 2 μg of Fe^{2+} (10 μg of $FeSO_4 \cdot 7H_2O$ per ml) were added. The final volume of medium in each plate (diameter, 9 cm) was 20 ml. In the agar medium, 0.3 μg of Fe^{2+} per ml was enough to inhibit toxin production (halo formation) by C7(β). In both liquid medium and agar medium, an excess iron concentration was 2 μg of Fe^{2+} per ml, about seven times as much as the minimum concentration of iron that inhibited toxin production by C7(β).

Bacterial growth. Bacterial growth was followed by measuring the optical density at 590 nm in a Bauch & Lomb spectrophotometer. One optical density unit was equivalent to about 10^9 colony-forming bacteria per ml.

Toxin production. Toxin production by growing cultures was achieved by incubation in modified Linggood-Fenton medium or PGT medium at 35°C in a rotary shaker at 180 rpm for about 24 h, and that under growth-limiting conditions was achieved by incubation in CST-6 medium at 35°C in a rotary shaker at 140 rpm for 2 h.

L-[^{3}H]tryptophan. L-[^{3}H]tryptophan (specific activity, 5.6 mCi/mmol) was obtained from New England Nuclear Corp. Radioactivity was measured in a Beckman liquid scintillation counter.

Radioactivity of toxin. The radioactivity of the toxin was assayed with specific precipitates obtained by a quantitative precipitation reaction with diphtheria antitoxin. Five-milliliter portions of radioactive culture filtrate were dialyzed in the cold against tris(hydroxymethyl)aminomethane-buffered saline (pH 7.5) for 3 days to remove free [^{3}H]tryptophan. After dialysis, an appropriate amount of unlabeled diphtheria toxin was added to each filtrate to make a total of about 100 flocculating units, and then excess (120 U) diphtheria antitoxin was added. The mixture was incubated at 35°C for 1 h and then placed in the cold for 3 days. The resulting precipitates were then washed twice with saline and dried for measuring.

Determination of the iron-binding capacities. Iron-deficient cell suspensions in 0.025 M maleate buffer (pH 6.8) and iron solution were mixed and incubated at 37°C for 60 min. After centrifugation, the iron contents of supernatants and precipitated cells were determined by using 10% ascorbic acid and *o*-phenanthroline as described by Yoneda and Ishihara (33).

Anti-β-phage serum. β^{hv} phages (15) were purified by ultracentrifugation and injected to rabbits. The serum value of the antiphage rabbit serum obtained was about 17.

Assay of diphtheria toxin. Toxin was determined by the Ramon et al. flocculation method (26). Toxicity was measured quantitatively by the rabbit skin reaction test, using diphtheria toxin no. 108 from the Vaccine Production Plant of our Institute as a standard.

SDS gel electrophoresis. Sodium dodecyl sulfate (SDS) gel electrophoresis was carried out as described by Weber and Osborn (32), using 10% gels.

Assay of adenosine 5′-diphosphate-ribosylating activity. Nictinamide adenine dinucleotide:elongation factor 2-adenosine 5′-triphosphate-ribose-transferase activity was measured by the methods of Gill and Pappenheimer (8). The activities of extracts of SDS polyacrylamide gel slices were assayed directly, without preliminary activation with trypsin.

RESULTS

Isolation and selection of bacterial mutants. Cells from exponentially growing cultures of strain C7(β) in modified Linggood-Fenton medium were collected and washed once with sterile saline. They were then suspended in 0.05 M tris(hydroxymethyl)aminomethane-malate buffer (pH 6.0) (1) at a cell density of approximately 4×10^8/ml and treated with 10 μg of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co.) per ml at 37°C. After incubation for 15 min, the bacteria were collected, washed with medium, resuspended in fresh medium, and incubated for 2 h at 35°C. The survivors (ca. 10%) were plated on tryptose agar plates containing excess iron (2 μg Fe^{2+} per ml) and horse antitoxin (1.5 U/ml). The plates were incubated at 35°C for 2 days, and colonies surrounded by a halo were selected.

In Fig. 1, the arrow indicates a colony that produced toxin in medium containing excess iron. This colony was picked and subcultured several times in modified Linggood-Fenton medium containing 2 μg of iron (Fe^{2+}) per ml. It was then plated on antitoxin-tryptose agar plates with excess iron. All colonies showed halo formation due to the toxin-antitoxin complex. In this way, five mutants producing toxin in medium with excess iron were obtained as homogeneous strains from about 40,000 colonies. The mutants were named C7hm722, C7hm723, C7hm726, C7hm728, and C7hm729.

Effect of iron on toxin production by mutants. Table 1 shows the effects of iron on toxin production by parent strain C7(β) and the five mutants under growing conditions. That of the parent strain C7(β) was inhibited completely by 0.3 μg of iron per ml in modified Linggood-Fenton and PGT media, but the mutants produced toxin at normal rates in both media even at 3 μg of iron per ml. Strains C7hm722, C7hm723, and C7hm728 produced as much or more toxin in excess iron as in iron-deficient medium. Toxin production by the other two strains, C7hm726 and C7hm729, was slightly inhibited by iron addition. Table 2 shows results of toxin production by the parent and mutant strains under growth-limiting conditions with succinate as the sole energy source. The strains were incubated, at 35°C in CST-6 medium at a cell density of optical density = 9.0, for 2 h. Under these conditions, parent strain C7(β) produced toxin in iron-depleted medium but not in the presence of excess iron. On the other hand, strain C7hm723 produced as much toxin in medium

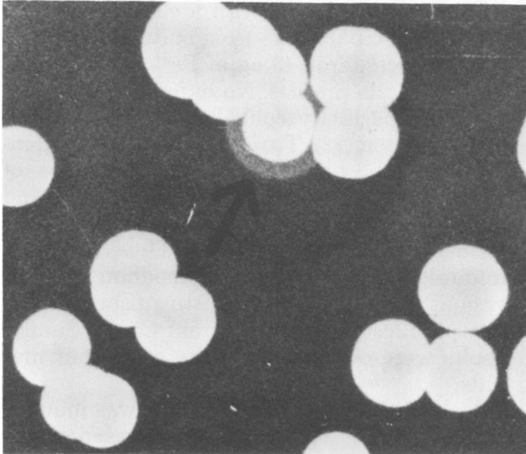

FIG. 1. *Selection of bacterial mutants by halo formation on an antitoxin agar plate. After nitrosoguanidine treatment, 0.1-ml portions (containing 100 to 200 surviving cells) of the appropriately diluted cultures were plated on agar plates containing 1.5 U of horse antitoxin per ml and excess iron (2 μg of Fe^{2+} per ml) and incubated at 35°C for 2 days. The arrow indicates a mutant colony producing toxin.*

TABLE 1. *Effect of iron on toxin production by C7(β) and mutant strains in modified Linggood-Fenton medium*

Strain	Fe^{2+} (0.1 μg/ml)		Fe^{2+} (3 μg/ml)	
	Toxin yield[a] (L_f/ml)	Final growth (OD)[b]	Toxin yield (L_f/ml)	Final growth (OD)
C7 (β)	20	15.3	0.1[c]	15.0
C7hm722	20–25	11.4	25	13.5
C7hm723	20–25	13.2	20–25	15.0
C7hm726	15–20	8.6	5–10	9.9
C7hm728	20–25	11.7	25–30	15.6
C7hm729	20	12.9	10–15	10.8

[a] Bacteria from exponentially growing cultures of C7(β) and mutant strains in iron-deficient medium were inoculated at a cell density of about 0.05 optical density unit into 10 ml of medium containing 0.1 or 3 μg of Fe^{2+} per ml in 100-ml Erlenmeyer flasks. The flasks were incubated at 35°C with rotary shaking at 180 rpm, and after 24 h the flocculation titers (L_f) of the culture supernatants were measured.

[b] OD, Optical density.

[c] Determined by the skin test, assuming 10^5 MRD/L_f.

with excess iron as the parent strain C7(β) did in iron-depleted medium. (The final pH of each culture was always more than 7.0.) Thus, results obtained under growth-limiting conditions are similar to those obtained with growing cultures.

Although treatment with nitrosoguanidine tends to affect adversely the growth rate of bacterial cells, the growth rates of strains C7hm723 and C7(β) were almost the same. This permitted toxin production by growing cultures of both strains to be compared in PGT medium at various iron concentrations (Table 3). Toxin production by parent strain C7(β) was almost completely inhibited by ≧0.3 μg of Fe^{2+} per ml, but that of strain C7hm723 was not influenced by the iron concentrations employed. However, it is not clear whether this phenotype of the mutant results from single mutation.

Ordinarily, toxin is only produced and released extracellularly in significant amounts by toxigenic strains of *C. diphtheriae* during the declining phase of growth after iron becomes limiting. Strain C7hm723 produced toxin in good yield during exponential growth, even at an iron concentration 30 times that sufficient to almost completely inhibit its production by C7(β) (Fig. 2). The differential rate of toxin synthesis was roughly constant.

When Fe-deficient bacterial suspensions were incubated for a short period in maleate buffers containing ferrous sulfate, the cells rapidly took

TABLE 2. *Effect of iron on toxin production by C7(β) and mutant strains under growth-limiting conditions*

Strain	Final yield of toxin[a] (L_f/ml)	
	No iron added	Fe^{2+} added (2 μg/ml)
C7 (β)	4	<0.1[b]
C7hm723	4	4
C7hm726	6	4

[a] About 50 ml of an exponential culture (optical density = 1.8) was centrifuged and the bacteria were suspended in 10 ml of CST-6 medium to an optical density of 9.0 in 100-ml Erlenmeyer flasks. The flasks were incubated at 35°C with rotary shaking at 140 rpm for 2 h. L_f, Flocculation unit.

[b] Calculated by the skin test.

TABLE 3. *Effect of iron on toxin production by strains C7(β) and C7hm723 in PGT medium*

Fe^{2+} added (μg/ml)	C7(β)		C7hm723	
	Toxin yield[a] (L_f/ml)	Final growth (OD)[b]	Toxin yield (L_f/ml)	Final growth (OD)
0.1	4	5.1	4–6	6.6
0.3	<0.1[c]	6.6	4–6	6.4
0.5	<0.1	6.6	4–6	6.3
1.0	<0.1	8.0	4–6	6.0
2.0	<0.1	8.0	4–6	6.4

[a] Exponentially growing cultures of C7(β) and C7hm723 in iron-deficient medium were centrifuged, and the cells were suspended at a density of 0.1 optical density unit in 10 ml of PGT medium containing various concentrations of iron in 100-ml Erlenmeyer flasks. The flasks were incubated as described in footnote *a* of Table 1. L_f, Flocculation unit.

[b] OD, Optical density.

[c] Calculated from the skin test.

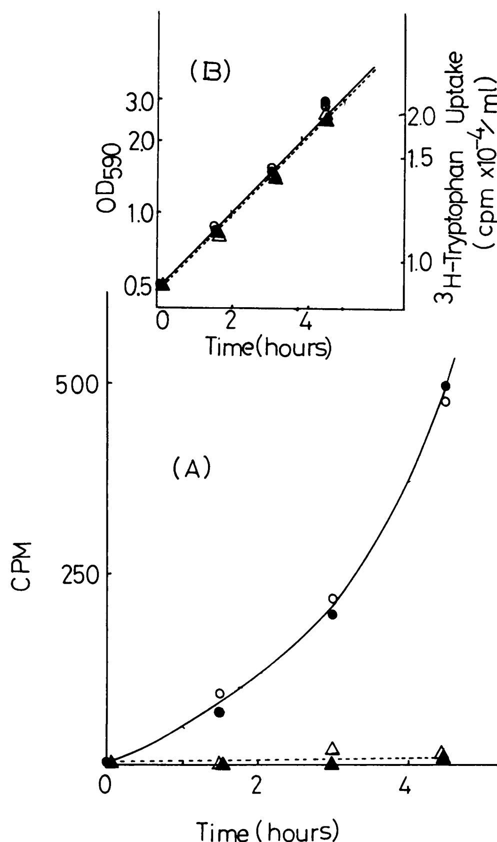

FIG. 2. *Incorporation of [^{3}H]tryptophan into toxin protein released extracellularly by parent and mutant cells under exponentially growing conditions in PGT medium. Bacteria from exponentially growing cultures of C7(β) and C7hm723 in iron-deficient medium were inoculated at a cell density of about 0.5 optical density unit (OD) in PGT medium containing 10 μg [^{3}H]tryptophan (50 μCi/ml) and either 0.1 μg (Δ, C7(β); O, C7hm723) or 3 μg (▲, C7(β); ●, C7hm723) per ml of iron (Fe^{2+}). The flasks were incubated at 35°C with rotary shaking at 180 rpm, and 5-ml portions of the culture were taken at 1.5-h intervals for measuring the toxin radioactivity. (A) Toxin production; 480 cpm = ca. 5 × 10^5 MRD. (B) Growth curve.*

up iron (Table 4). Even at relatively high iron concentrations (2 μg of Fe^{2+} per ml), all of the strains tested bound more than 60% of the added iron. Although the wild-type C7(β) strain appeared to bind somewhat more iron per cell at these high concentrations than did the mutants, the latter produced toxin at a maximum rate even when their bound-iron content was more than five times that of inhibited C7(β).

Characterization of toxin from mutant. Proteins in the supernatants of cultures in PGT medium were precipitated with 70% $(NH_4)_2SO_4$ and then examined by SDS gel electrophoresis. Figure 3 shows results with a culture of strain C7hm723. Preparations from cultures with and without excess iron (3 μg of Fe^{2+} per ml) gave similar protein profiles. No difference in immunological specificity could be detected by immunodiffusion (22). Toxicities (MRD) and adenosine 5′-diphosphate-ribosylating activities of the toxins produced by strain C7hm723 and parent strain C7(β) were almost the same on the basis of flocculation units. Thus, the toxin produced by the mutant in medium with or without excess iron was indistinguishable from that produced by parent strain C7(β). Similar results were obtained with the other four mutants.

Each of the five mutant strains was induced with ultraviolet light, and the released phage was used to lysogenize C7(−). A lysogen was isolated for each phage which could not be distinguished from C7(β) and was inhibited by excess iron. These results suggested that each of the five mutants carries a mutation in the bacterial genome which is involved in control of *tox* gene expression.

Superinfection of a mutant strain with β45c phage. To confirm that the mutant strain resulted from a mutation of a bacterial gene, mutant C7hm723 cells were superinfected with the clear-plaque-forming β45c phage at a multiplicity of 2 (9) and cultured in the presence of 3 μg of Fe^{2+} per ml at 35°C for 2 h. The extracellular proteins produced by the β45c-superinfected C7hm723 strain in PGT medium containing excess iron were compared with those produced by β45c-superinfected strain C7(β) or

TABLE 4. *Iron-binding capacities of iron-deficient cells of C7(β) and mutants in maleate buffer*

Fe^{2+} concn in medium (μg/ml)	Strain	Fe^{2+} bound to cells[a] (μg/ml)	Ratio of cell-bound iron/external iron
0.3	C7(β)	0.25	0.83
	C7hm722	0.24	0.80
	C7hm723	0.20	0.67
	C7hm728	0.22	0.73
1.0	C7(β)	0.79	0.79
	C7hm722	0.69	0.69
	C7hm723	0.56	0.56
	C7hm728	0.56	0.56
2.0	C7(β)	1.71	0.85
	C7hm722	1.31	0.65
	C7hm723	1.21	0.60
	C7hm728	1.21	0.60

[a] An iron-deficient cell suspension (4 × 10^9 cells per ml) of C7(β) or of a mutant strain was incubated in maleate buffer containing various amounts of iron at 35°C for 60 min.

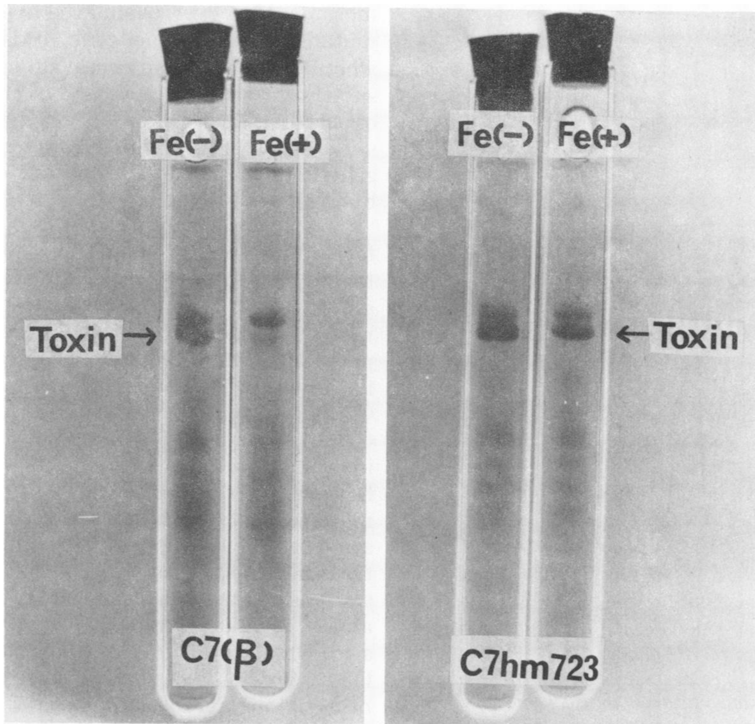

FIG. 3. *SDS gel patterns of proteins from crude culture supernatants of strain C7hm723. The cells were cultured in PGT medium containing 0.1 μg of Fe^{2+} per ml (Fe−) and 2 μg of Fe^{2+} per ml (Fe+) at 35°C for 24 h. The supernatants of both cultures were concentrated about 40-fold, and 20 μl of each was then subjected to SDS gel electrophoresis. SDS gel patterns of extracellular proteins from wild-type C7(β) are shown for comparison.*

C7(β-723) under similar conditions. The latter strain was the C7(−) strain lysogenized with phage derived from strain C7hm723. Figure 4 shows the patterns of these proteins upon SDS-gel electrophoresis. Two major bands at positions corresponding to molecular weights of 62,000 and 45,000 that had adenosine 5′-diphosphate-ribosylating activity are seen in the preparation from the supernatant of β45c-superinfected C7hm723 strain, whereas neither protein was produced by β45c-superinfected C7(β-723) strain. Presumably the toxin band shown in Fig. 4 was largely "nicked," since intact toxin has no enzymatic activity (5, 8). The protein band of fragment A (24,000 daltons [4, 8]), which had enzymatic activity was also observed in the supernatant of β45c-superinfected C7hm723, and was degraded from CRM45 and toxin. Since the other protein bands are observable with extracellular proteins of strain C7(−) cultured in medium with and without excess iron, these proteins were neither phage- nor iron-dependent.

Elimination of prophage from mutant C7hm723. To establish further that the mutant strain carries a mutation in the bacterial genome which is involved in control of *tox* gene expression and develop a procedure for toxin or mutant protein production in nondeferrated media, we tried to eliminate prophage from mutant C7hm723.

Cells from cultures of mutant C7hm723 in exponential growth were collected and induced by irradiation with an ultraviolet dose sufficient to kill about 90% of the cells. The surviving cells were plated on tryptose agar plates containing excess iron, horse antitoxin, and anti-β-phage serum. After a 2-day incubation, colonies without haloes of toxin-antitoxin precipitate were selected (Fig. 5) and tested for toxin production, phage liberation, and sensitivity to β phage. One cured strain, C7hm723(−), was isolated from about 10^4 colonies.

The cured strain was lysogenized with β, β197, or β-NG2. Toxin or cross-reacting material was produced by each of the new lysogenic strains at almost the same rate as that by parent C7hm723 in medium with or without excess iron.

From these results we conclude that the bacterial host factor is essential, at least in part, for inhibition of toxin production by iron. The

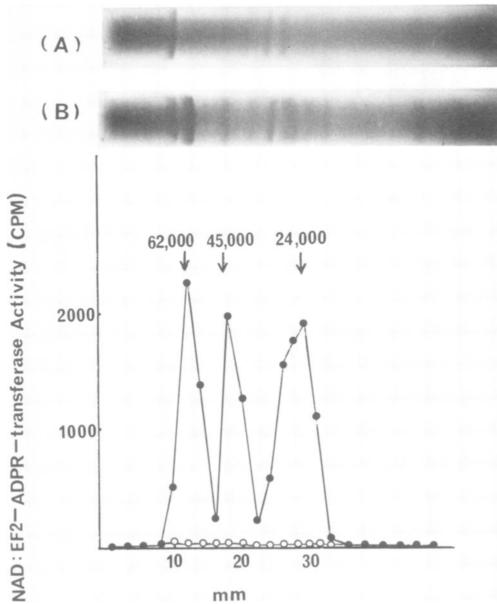

FIG. 4. *SDS gel patterns of proteins from crude culture supernatants of strain C7(β-723) (A) and strain C7hm723 (B) superinfected with β45c phage. The cells were each cultured in PGT medium containing excess iron (3 μg of Fe^{2+} per ml) and were superinfected with β45c phages (multiplicity of infection of 2) as described in the text. After absorption, the cells were collected, washed, and suspended to an optical density of 4.0 in PGT medium containing excess iron. After 2 h of incubation at 35°C, the cells were removed. The supernatants of both cultures (A and B) were concentrated about 100-fold, and 20 μl of each was subjected in duplicate to SDS gel electrophoresis. One each of the gels was stained, and the other was analyzed for adenosine 5'-diphosphate-ribosylating activity after elution of 2 mm slices. Symbols: ○, (A); ●, (B). NAD, Nicotinamide adenine dinucleotide.*

cured strain C7hm723(−) is useful for the production of cross-reacting material using medium without deferration.

DISCUSSION

It is clear that the structural information for diphtheria toxin synthesis is carried by corynebacteriophages, such as temperate phage β, and that the *tox* gene expression is independent of all other phage gene products identified thus far. The control mechanisms of *tox* gene expression were studied in the present work. Our results show that *C. diphtheriae* C7 carries a gene(s) involved in regulation of *tox* gene expression.

Five mutant strains were isolated. However, it is not clear whether they have a mutation in the same or different genes of the bacterial chromosome. Moreover, even if the five mutants are mutated in the same gene, it is not known whether the mutation occurs on the same gene site. From an understanding of the properties of the bacterial factor, it will be possible to understand part of the characteristics of the genetic alteration of the mutant cells isolated by us.

The bacterial factor is apparently not essential for cell growth. As shown in Table 1, each of the mutant strains, especially C7hm723, can grow well at low iron concentrations. *E. coli* mutants carrying mutations in their iron transport system require high concentrations of iron for growth (31). The amount of iron binding to C7(β) was slightly greater than that binding to C7hm723 when the two strains were incubated in same concentration of iron. At this time, however, it is still difficult to say whether our mutants carry mutations in an iron transport system. The amounts of iron bound by the mutants became greater with increasing of concentrations of iron, while the cell growth remained almost constant. There is presently no evidence as to whether this excess iron is stored intracellularly or whether it is merely surface bound to bacterial membranes or cell walls.

Recently, Murphy et al. (21) reported the isolation of a mutant β phage lysogen, C7 ($\beta^{tox^+ \, ct1}$), which is relatively insensitive to the iron-mediated inhibition of diphtheria toxin production. Their evidence suggests that the genetic

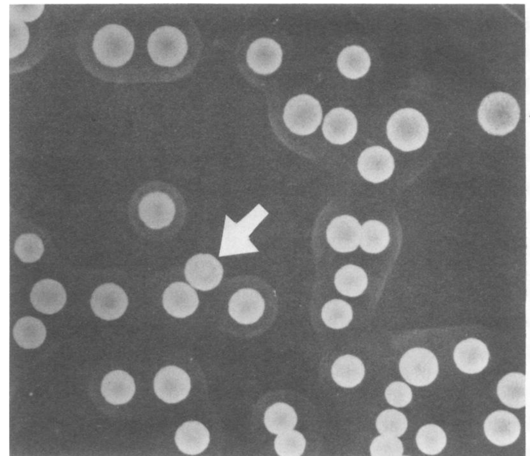

FIG. 5. *Selection of cured strain from a C. diphtheriae mutant by using antitoxin plates. After ultraviolet irradiation, surviving cells (100 to 200) were plated on antitoxin plates containing 1.5 U of horse antitoxin per ml, 2 μg of Fe^{2+} per ml, and anti-β-phage serum (serum value, 17) and incubated at 35°C for 2 days. Colonies not encircled by a halo were then picked up. The arrow indicates a cured colony not producing toxin.*

defect lies within the *tox* operator region and is not caused by a mutation in the *tox* structural gene itself. It seems reasonable to conclude that there is a bacterial host protein that can repress expression of the phage *tox* operon. Ferrous iron would be required for its binding to the *tox* operator gene. Presumably, our mutant bacterial strains produce an altered gene product that no longer can bind tightly to the *tox* operator even when iron is present in excess. Similarly, the *tox* operator gene mutants studied by Murphy et al. (21) bind normal repressor poorly even under optimal iron concentrations.

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