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Regulation of Toxinogenesis in *Corynebacterium diphtheriae*

I. Mutations in Bacteriophage β That Alter the Effects of Iron on Toxin Production

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Diphtherial toxin is produced in maximal yields by *Corynebacterium diphtheriae* C7(β^{tox^+}) only when iron is present in growth-limiting amounts. Toxin production is markedly decreased under high-iron conditions. We studied the role of the bacteriophage β genome in this apparent regulation of toxin production by iron. Using a passive immune hemolysis assay to detect toxin antigen production in individual plaques, we identified rare phage mutants that were toxinogenic in high-iron medium. Lysogenic derivatives of *C. diphtheriae* C7 harboring such phage mutants were constructed. The lysogens were compared with wild-type strain C7(β) for their ability to produce toxin in deferrated liquid medium containing varying amounts of added iron. Quantitative tests for extracellular toxin were performed by competitive-binding radioimmunoassays. We identified phenotypically distinct mutant strains that produced slightly, moderately, or greatly increased yields of toxin antigen under high-iron conditions. The toxin produced by the mutant lysogens was biologically active and immunochemically indistinguishable from wild-type toxin. Complementation experiments demonstrated that the phage mutation designated *tox*-201 had a *cis*-dominant effect on the expression of the toxin structural gene of phage β. The characteristics of the *tox*-201 mutation suggest that it defines a regulatory locus of phage β that is involved in control of toxinogenesis by iron in *C. diphtheriae*.

The production of diphtherial toxin by *Corynebacterium diphtheriae* is controlled by bacteriophage conversion (5, 11, 12, 15). The structural gene for toxin is present in several corynebacteriophages, including the well-studied phage β (37, 55). Many nontoxinogenic mutants of β have been isolated and characterized (18, 26, 32, 55, 57, 58). The toxin structural gene has been mapped in relation to other genes of phage β (19, 50), and fine-structure mapping within the *tox* locus has been reported (18, 27). Toxin can be produced when phage β is present in *C. diphtheriae* as a vegetative phage (30), as a prophage (5, 13), or as a nonreplicating exogenote in cells with lysogenic immunity (13). Nontoxinogenic mutants of phage β are normal with respect to vegetative and lysogenic phage functions other than toxin production (18, 26, 32, 57). Thus, the expression of the toxin structural gene is controlled independently of other known phage functions.

Genetic regulation of toxinogenesis in *C. diphtheriae* involves both the bacterium and the bacteriophage. Maximal toxin yields can vary greatly when specific tox^+ corynebacteriophages are present in different host strains of *C. diphtheriae* (3, 31). Several investigators demonstrated that some phages isolated from nontoxinogenic strains of *C. diphtheriae* were tox^+ in other *C. diphtheriae* host strains (16, 43). Bacha and Murphy showed recently that toxin was produced when specific *tox* mutants of phage β infected *C. diphtheriae* host strains carrying appropriate nonsense suppressor alleles (2). Mutations associated with decreased toxin yields have been described both in corynebacteriophage β (56) and in its usual laboratory host, *C. diphtheriae* C7 (40, 57).

It was established nearly 50 years ago that toxin production by *C. diphtheriae* is influenced dramatically by the amount of iron in the growth medium (29, 42, 44). Toxin production is maximal under conditions of iron starvation and is significantly less than maximal under high-iron conditions in all of the toxinogenic strains of *C. diphtheriae* studied (10, 30, 34, 39, 57). Many biochemical and ultrastructural correlates of iron deficiency in *C. diphtheriae* have been described, but the mechanisms directly responsible for decreased toxin yield under high-iron conditions are not well defined (4, 39, 41).

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Recently, several investigators have used genetic techniques to study the relationships between iron and toxinogenesis in *C. diphtheriae*. Murphy et al. isolated the bacteriophage mutant $\beta^{tox^+}_{cI1}$ and demonstrated that toxin production by the C7($\beta^{tox^+}_{cI1}$) lysogen was partially resistant to the inhibitory effect of iron (38). Kanei et al. isolated a mutant called C7(β)hm723 that produced as much toxin in high-iron medium as wild-type C7(β) produced in low-iron medium (23). They demonstrated that the mutation responsible for this altered phenotype of C7(β)hm723 was in the genome of the C7 bacterial host. Additional bacterial mutants that produce high yields of toxin under high-iron conditions have been isolated and are being characterized in our laboratory (S. J. Cryz, Jr., and R. K. Holmes, manuscript in preparation). Based in part on the properties of the mutants described above, Murphy and his collaborators postulated that the toxin structural gene in phage β is part of a *tox* operon, that the *tox* operon is negatively regulated by a specific repressor, that the bacterial genome codes for a protein aporepressor, and that the specific repressor is a complex of the aporepressor with iron (35, 38). Additional studies will be required either to confirm or to disprove this hypothesis.

In the present report we describe the isolation and preliminary characterization of additional β phage mutants that permit increased amounts of toxin to be produced by infected cells under high-iron conditions. Our long-range goals are to use phenotypically distinctive *tox* regulatory mutants of phage β and of *C. diphtheriae* C7 to analyze the molecular mechanisms that mediate the effects of iron on toxinogenesis.

(A preliminary report of these results was presented at the 80th Annual Meeting of the American Society for Microbiology, 11-16 May 1980, Miami Beach, Fla.)

MATERIALS AND METHODS

Bacteria, bacteriophages, and conditions of cultivation. *C. diphtheriae* $C7_s(-)^{tox^-}$ (hereafter designated C7) is nontoxinogenic, is sensitive to phage β^{tox^+}, and is the usual indicator strain and propagating host for the corynebacteriophages used in this study (18, 19). The phage strain $\beta^{tox\text{-}2}$ is a mutant of β that codes for a nontoxic 26,000-molecular-weight protein (cross-reacting material [CRM26]) corresponding to an amino-terminal fragment of diphtheria toxin (18). Isolation of phage $\beta^{tox\text{-}201}$ is described below, and construction of the recombinant phage $\beta^{h\ tox\text{-}201\ tox\text{-}2\ h'}$ is described elsewhere (63). Phage $\beta^{tox^+}_{cI1}$ was provided by J. R. Murphy in the lysogenic strain C7($\beta^{tox^+}_{cI1}$) (38). Procedures for routine cultivation of bacterial strains, for propagation and assay of bacteriophages, and for construction of lysogenic derivatives of C7 have been described previously (18, 19). Strains of *C. diphtheriae* to be tested quantitatively for production of toxin or related CRMs were grown with rotary shaking for 18 h at 37°C in 10-ml portions of deferrated PGT-maltose medium to which iron was added at concentrations from 0 to 1.6 μg of Fe^{2+} per ml (20). Culture supernatants were collected, sterilized by filtration through membrane filters (type GA; pore diameter, 0.45 μm; Gelman Sciences, Inc., Ann Arbor, Mich.), stored at 4°C, and tested within 24 h for toxin or CRM by appropriate radioimmunoassays. In experiments on the time course of growth and toxin production, flasks containing deferrated PGT-maltose medium with 0.075, 0.3, or 1.0 μg of added Fe^{2+} per ml were inoculated with samples from iron-starved cultures of appropriate *C. diphtheriae* strains and were incubated as described above. Samples were removed at intervals for measurements of absorbance at 590 nm and of extracellular toxin.

Diphtherial toxin, toxoid, and antitoxin. Purified diphtherial toxin Dc202, purified fragment A, and formolized toxoid prepared from Dc202 toxin have been described previously (8). A goat was immunized with Dc202 toxoid, and immunoglobulin G containing 25 antitoxin units (A.U.) per mg was prepared from the goat antiserum (62). Hyperimmune rabbit antiserum R21 containing 600 A.U./ml was raised against toxoid prepared from a sample of purified unnicked diphtherial toxin Jn141 (8).

Tests for diphtherial toxin. (i) Passive immune hemolysis assay. The diphtherial toxin antigen produced in individual plaques of corynebacteriophages was detected by using the passive immune hemolysis assay method described elsewhere (62).

(ii) Elek tests. Qualitative tests for production of toxin antigen by lysogenic *C. diphtheriae* strains were performed by a modification of the method of Elek (18). DA-1 medium (62) was substituted for the medium used in our earlier studies (18). For some experiments, DA-1 medium was supplemented with varying concentrations of ferrous iron, as indicated below.

(iii) Radioimmunoassays. Competitive-binding radioimmunoassays for toxin antigen or for related nontoxic CRMs in supernatants from cultures of *C. diphtheriae* were developed in our laboratory and were performed as described elsewhere (8). The assays for toxin used R21 antitoxoid and ^{125}I-labeled toxin. CRM26, which is encoded by the *tox*-2 allele in phage β, did not cross-react substantially in this assay but could be detected in a second competitive-binding radioimmunoassay in which R21 antitoxoid and ^{125}I-labeled fragment A were used. Toxin could be measured either in the presence or in the absence of CRM26, whereas specific assays for CRM26 were performed only with culture supernatants that did not also contain toxin (8).

(iv) Toxicity tests. The toxicities of various strains of *C. diphtheriae* were determined in rabbits by intracutaneous tests (18, 19).

Methods for induction of mutants in phage β. (i) Mutagenesis with ethyl methane sulfonic acid ester. Samples containing 5-ml portions of phage β stocks (0.1×10^{11} to 2×10^{11} PFU/ml) were centrifuged for 90 min at 19,000 × *g* and 4°C. Supernatants were discarded, and the phage pellets were suspended in 1.0-ml volumes of 0.2 M Tris-chloride buffer (pH 7.5). Samples containing 2 ml of 0.02 M ethyl methane sulfonic acid ester in 0.2 M Tris buffer (pH 7.5) were

added to the 1-ml phage suspensions. After incubation at 37°C for 60 min, samples were diluted 10-fold in 0.2 M Tris buffer (pH 7.5), and phage assays were performed. These conditions resulted in effective mutagenesis with little inactivation of infectivity.

(ii) Mutagenesis with nitrous acid. Phage were treated by a modification of the method of Bautz-Freese and Freese (6). Phage from 11.5-ml volumes of phage β stocks were collected by centrifugation as described above and were suspended in 1.5-ml portions of TEN buffer (10 mM Tris-chloride, 1 mM EDTA, 0.25 M NaCl, pH 8). After samples were removed for phage assays, a 1.5-ml portion of freshly prepared, filter-sterilized 0.2 M $NaNO_2$ in 0.5 M sodium acetate buffer (pH 4.2) was added to each phage suspension. The mixtures were incubated for 10 min at room temperature, diluted fivefold into 0.5 M Tris-chloride buffer (pH 8.2) containing 1 mM $MgSO_4$, sterilized by membrane filtration, and assayed for infectious phage. This treatment reduced infectivity by 90 to 99%.

(iii) Mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at concentrations of 25 to 90 μg/ml was performed by a minor modification of method II of Holmes and Barksdale (19).

Screening for *tox* regulatory mutants of phage β. The passive immune hemolysis assay (62) was used to screen for mutant β phages that permitted detectable quantities of toxin to be produced during formation of plaques in high-iron medium. Deferrated agar (DA-1 and DA-0.35 [62]) was supplemented with 0.8 or 1.6 μg of Fe^{2+} per ml, and the mutagenized phage stocks were diluted and assayed to yield approximately 1,000 plaques per plate. Plaques of wild-type phage β in deferrated agar medium with no added iron served as positive controls for detecting toxin antigen by the passive immune hemolysis assay. The C7 indicator cells which were used for assays of mutagenized or control phage stocks were grown in low-iron medium. Rare mutant plaques exhibiting small hemolytic halos in the high-iron agar medium were picked and assayed under routine conditions to recover viable phage. Inocula picked from individual turbid plaques were examined qualitatively for toxin production in Elek tests in media containing 0 to 3.2 μg of added Fe^{2+} per ml in increments that did not exceed twofold dilutions. C7(β^{tox+}) and C7 were included as positive and negative controls for the Elek tests. We identified phage mutants that gave positive Elek tests in media containing sufficient iron so that controls with C7(β^{tox+}) were negative. These mutants were purified by repeated single-plaque isolations and were saved for further characterization.

Complementation tests. All reagents were prepared in acid-cleaned glassware with quartz-distilled water. The strains of *C. diphtheriae* used for complementation tests were cultivated in deferrated PGT-maltose medium containing 0.075 μg of added Fe^{2+} per ml for 18 h; 1-liter flasks containing 100-ml volumes of deferrated PGT-maltose medium supplemented with 0.075 μg of Fe^{2+} per ml (low iron) or 1.0 μg of Fe^{2+} per ml (high iron) were inoculated with samples from the low-iron 18 h cultures to give approximately 5×10^7 colony-forming units per ml and were incubated with shaking at 37°C until mid-log phase (absorbance at 590 nm, 0.5; 2×10^8 to 4×10^8 colony-forming units per ml). Samples were removed for viable counts, and 20-ml volumes of the cultures were transferred into 125-ml flasks and then superinfected with phages or left uninfected as controls. The superinfecting phage stocks had been freshly prepared the previous day, deferrated by incubation overnight at 4°C with sterile Chelex 100 resin (2 g/10 ml; Bio-Rad Laboratories, Richmond, Calif.), filtered, and titrated for infectivity. The superinfecting phages were added at multiplicities of approximately 20 PFU/colony-forming unit and were allowed to adsorb for 20 min. Samples removed and assayed at that time typically showed that 80% or more of the input phage had been adsorbed. Sterile Tween 80 (Sigma Chemical Co., St. Louis, Mo.) was added to each flask at a final concentration of 0.2% to prevent further phage absorption (17). The cultures were then incubated for 18 h at 37°C with shaking. Absorbance of the cultures at 590 nm was measured, and culture supernatants were collected, sterilized by membrane filtration, and tested for diphtherial toxin or CRMs by appropriate radioimmunoassays. The superinfecting phage stocks were also tested by radioimmunoassays to determine how much preformed toxin they contained, and the small amounts of toxin antigen in the superinfecting phage stocks were subtracted as background from the results obtained in the complementation experiments.

RESULTS

The addition of excess iron to cultures of *C. diphtheriae* C7(β) decreases the yield of diphtherial toxin. To investigate the role of phage β in this phenomenon, we characterized mutations in β that were associated with increased production of toxin during infection of *C. diphtheriae* in high-iron medium.

Stocks of β phage were treated with various chemical mutagens. To document the effectiveness of mutagenesis, we determined the frequencies of clear plaque mutants in the mutagenized phage stocks. Spontaneous clear plaque mutants are very rare and were not observed in this study or in our previous studies (18, 19). The frequencies of clear plaque mutants per viable phage after chemical mutagenesis were as follows: *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, 2.5×10^{-3} to 4×10^{-3}; ethyl methane sulfonic acid ester, 0.6×10^{-3} to 1.5×10^{-3}; and nitrous acid, 0.6×10^{-3} to 1.5×10^{-3}. The mutagenized β phage stocks were screened for *tox* regulatory mutants by using our passive immune hemolysis assay in high-iron medium (62). We looked for the rare plaques that were surrounded by hemolytic halos, indicating production of detectable amounts of toxin antigen under these conditions. The results of these experiments are summarized in Table 1.

After mutagenesis of phage β with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, ethyl methane sulfonic acid ester, or nitrous acid, *tox* regulatory mutants were observed at frequencies of 6×10^{-5} to 24×10^{-5} per viable phage. Inocula from

TABLE 1. *Isolation of tox regulatory mutants of phage β*

Phage strain	Mutagenic agent	No. of independent expt	Qualitative tests for production of toxin in high-iron medium	
			Passive immune hemolysis test (no. of hemolytic plaques/total no. of plaques)[a]	Elek test: minimum concn of Fe^{2+} required for negative test (μg/ml)[b]
β^{tox+}	—[c]	—	—	0.08
β^{tox+}_{ct1}	—	—	—	0.08
$\beta^{tox-201}$	NTG[d]	4	5/24,600	1.2
$\beta^{tox-202}$	NTG			0.4
Three mutants[e]	NTG			0.2
$\beta^{tox-203}$	EMS[f]	4	7/110,000	0.2
Six mutants[e]	EMS			0.2
Seven mutants[e]	Nitrous acid	3	7/29,800	0.2

[a] Mutants were screened by using the passive immune hemolysis plaque assay. Media were supplemented with iron to yield a final concentration of 0.8 or 1.6 μg of Fe^{2+} per ml.

[b] Deferrated medium was supplemented with iron at concentrations of 0 to 3.2 μg of added Fe^{2+} per ml.

[c] —, C7 (β^{tox+}) and C7(β^{tox+}_{ct1}) were tested as controls for toxin production in the passive immune hemolysis assays and Elek tests. Hemolytic plaques were never observed surrounding plaques of wild-type C7(β^{tox+}) in numerous experiments done under high-iron conditions.

[d] NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

[e] These mutants were not assigned specific strain designations. $\beta^{tox-203}$ was considered to be representative of this phenotypic class of mutants.

[f] EMS, Ethyl methane sulfonic acid ester.

wild-type and mutant plaques were compared by Elek tests in media supplemented with varying quantities of iron. In this manner, we identified three phenotypically distinct classes of mutants that showed markedly, moderately, and slightly increased resistance to the inhibitory effects of iron on toxinogenesis. Phages $\beta^{tox-201}$, $\beta^{tox-202}$, and $\beta^{tox-203}$ were selected as representative strains for further studies.

Intracutaneous tests in rabbits demonstrated that C7 strains lysogenic for phages $\beta^{tox-201}$, $\beta^{tox-202}$, and $\beta^{tox-203}$ produced biologically active diphtherial toxin. Quantitative bioassays of culture supernatants demonstrated that these mutant strains produced at least as much toxin as wild-type C7(β).

We compared mutant lysogens with wild-type C7(β) in more detail by using competitive-binding radioimmunoassays for quantitative measurements of toxin. The mutant and wild-type strains were grown for 18 h in deferrated liquid medium supplemented with varying concentrations of iron. Figure 1A shows the results of a typical experiment with strains C7(β), C7(β^{tox+}_{ct1}), C7($\beta^{tox-201}$), C7($\beta^{tox-202}$), and C7($\beta^{tox-203}$). Figure 1B illustrates the reproducibility of results obtained in successive experiments with C7(β) and C7($\beta^{tox-201}$). With no added iron, bacterial growth was severely restricted (data not shown), and toxin production was poor. With 0.075 μg of added Fe^{2+} per ml, toxin production was maximal for each strain, and all of the mutant lysogens produced more toxin than the wild type. At higher concentrations of iron, all strains produced submaximal yields of toxin. The absolute amounts of toxin produced under high-iron conditions were reproducible but varied greatly from strain to strain. With wild-type C7(β) the average toxin yield was 5.7 μg/ml under optimal conditions and 0.024 μg/ml under high-iron conditions. In contrast, C7($\beta^{tox-201}$) produced 21 μg of toxin per ml under optimal conditions and 4.5 μg/ml under high-iron conditions. Thus, C7($\beta^{tox-201}$) produced almost 200 times more toxin than wild-type C7(β) under high-iron conditions.

The time course of toxin production in liquid medium containing varying concentrations of iron was studied with strains C7(β) and C7 ($\beta^{tox-201}$). Cultures were grown in deferrated PGT medium containing 0.075, 0.3, or 1.0 μg of added Fe^{2+} per ml, and samples were removed at intervals over 24 h to measure absorbance and extracellular toxin (Fig. 2 and 3). With wild-type C7(β) in low-iron medium, toxin accumulated rapidly after 9 h (Fig. 2A), during the final generations of bacterial growth (Fig. 3A). With C7($\beta^{tox-201}$) toxin was also produced rapidly after a lag of 9 h (Fig. 2B) during the final generations of bacterial growth (Fig. 3B). Although rela-

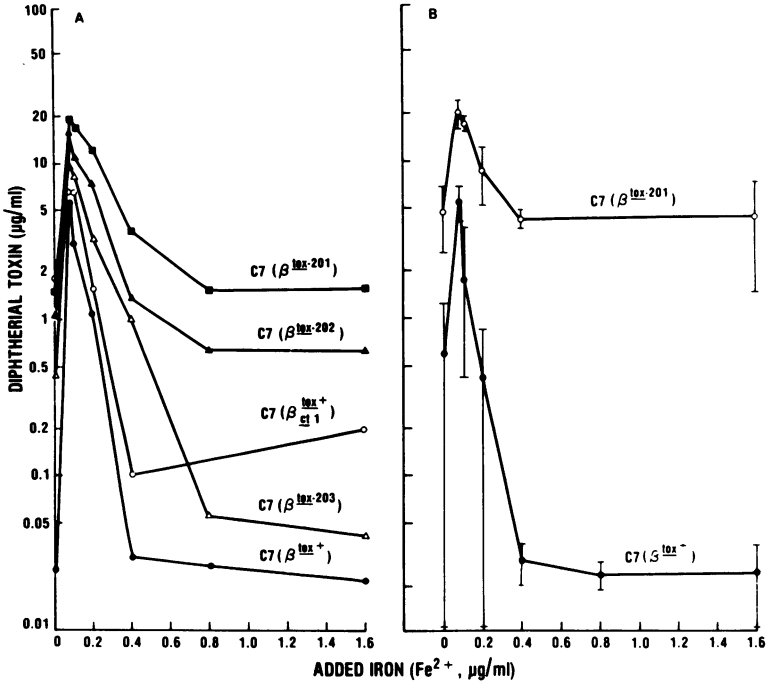

FIG. 1. *Production of toxin by C. diphtheriae in liquid cultures containing varying concentrations of ferrous iron. (A) Comparison of toxin production by C. diphtheriae strains C7(β^{tox^+}), C7($\beta^{tox\text{-}201}$), C7($\beta^{tox\text{-}202}$), C7($\beta^{tox\text{-}203}$), and C7($\beta^{tox^+}_{cl1}$) in 18-h cultures. Supernatants were collected and assayed for toxin by radioimmunoassay, as described in the text. (B) Reproducibility of toxin yields in cultures of C. diphtheriae C7(β^{tox^+}) (n = 5) and C7($\beta^{tox\text{-}201}$) (n = 3) containing varying concentrations of added Fe^{2+}. Bars indicate standard deviations.*

tively large amounts of toxin were produced by C7($\beta^{tox\text{-}201}$) under high-iron conditions, the differential rate of toxin production during the late log phase of bacterial growth was lower in the cultures with excess iron. Because C7($\beta^{tox\text{-}201}$) produced large amounts of toxin in high-iron medium only during late log phase, it seems probable that physiological changes associated with the terminal stages of growth are important for regulation of toxinogenesis in *C. diphtheriae*.

We designed a complementation system to analyze expression of the toxin structural gene in cells containing both wild-type and mutant alleles of the *tox*-201 locus. These experiments were based on the following two previous observations: (i) the CRM protein encoded by the *tox*-2 allele of the toxin structural gene can be easily distinguished from native diphtherial toxin by using specific radioimmunoassays (8); and (ii) different alleles of the toxin structural gene carried on prophage and superinfecting phage genomes can both be expressed in cells with lysogenic immunity (13). We prepared mid-log-phase cultures of lysogenic *C. diphtheriae* growing under defined low-iron or high-iron conditions, superinfected them with genetically marked β phages at sufficiently high multiplicities so that the cells would remain superinfected into stationary phase, and analyzed quantitatively the expression of the toxin structural gene alleles located *cis* or *trans* with respect to the *tox*-201 mutation. The results of this series of experiments are summarized in Table 2.

In control experiments without superinfecting phage, production of toxin by C7(β) and production of CRM by C7($\beta^{tox\text{-}2}$) were decreased to very low levels under high-iron conditions. In contrast, the relative yield of toxin from C7($\beta^{tox\text{-}201}$) and the relative yield of CRM from C7 ($\beta^{tox\text{-}201\ tox\text{-}2}$) were high under high-iron conditions. Toxin production in C7($\beta^{tox\text{-}2}$) superinfected with β^{tox^+} decreased markedly under high-iron conditions in a manner comparable to the C7(β) control (Table 2, experiments 1 and 2). In contrast, cultures of C7($\beta^{tox\text{-}2}$) superinfected with $\beta^{tox\text{-}201}$ produced half as much toxin as the C7($\beta^{tox\text{-}201}$) control cultures in both high-iron and low-iron medium (Table 2, experiments 2 and 3). Thus, *tox*-201 was dominant over the wild-type regulatory allele. This conclusion was confirmed by demonstrating that the high levels of toxin production characteristic of C7($\beta^{tox\text{-}201}$)

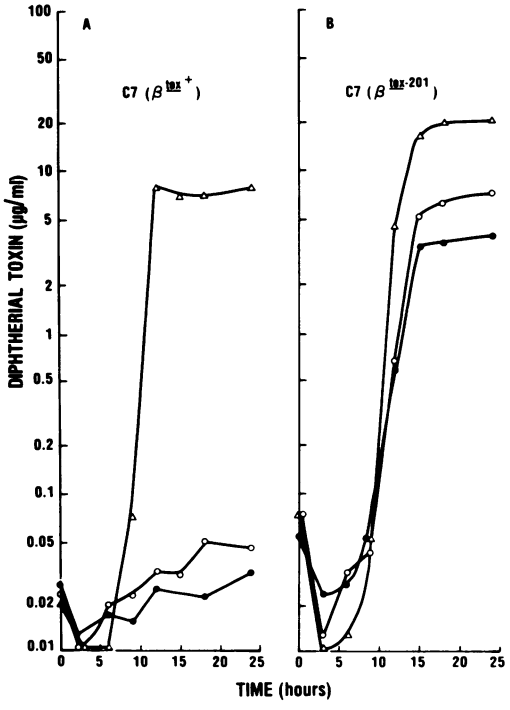

FIG. 2. *Time course of toxin production by C. diphtheriae in cultures containing varying concentrations of added iron. Cultures in deferrated liquid medium supplemented with 0.075 μg of added Fe^{2+} per ml (△), 0.3 μg of added Fe^{2+} per ml (○), or 1.0 μg of added Fe^{2+} per ml (●) were incubated, and samples of supernatants were tested for toxin as described in the text. (A) C7(β^{tox^+}). (B) C7($\beta^{tox\text{-}201}$).*

under high-iron conditions were maintained after superinfection with wild-type β^{tox^+} (Table 2, experiment 3). To determine whether dominance of *tox*-201 was expressed in the *cis* or *trans* orientation, we constructed a recombinant β phage carrying both *tox*-201 and *tox*-2 alleles and performed the experiments shown in Table 2, experiment 4. When C7($\beta^{tox\text{-}201\ tox\text{-}2}$) was superinfected with wild-type phage β, production of toxin decreased markedly under high-iron conditions in a manner similar to the wild-type C7(β) control (Table 2, experiments 1 and 4). Thus, the $\beta^{tox\text{-}201\ tox\text{-}2}$ prophage did not code for a diffusible positive regulator that stimulated high yields of toxin from the superinfecting β^{tox^+} phage under high-iron conditions. As expected however, the mutant phenotype of the superinfecting $\beta^{tox\text{-}201}$ phage was expressed in the C7($\beta^{tox\text{-}201\ tox\text{-}2}$) host (Table 2, experiments 2 and 4). We conclude that the altered regulatory phenotype controlled by the *tox*-201 mutation is expressed in a *cis*-dominant manner.

DISCUSSION

Many investigators have observed that production of diphtherial toxin by *C. diphtheriae* is decreased by excess iron in the culture medium (4, 29, 39, 41, 42, 44). For convenience, we refer to this phenomenon as the iron effect. To examine the role of the β phage genome in the iron effect, we isolated and characterized mutants that produced more toxin than wild-type phage β during growth in *C. diphtheriae* under high-iron conditions. Three phenotypically distinct classes of mutants were identified (Table 1). $\beta^{tox\text{-}201}$ was significantly more resistant to the iron effect than other mutants isolated in this study or $\beta^{tox^+}_{ct1}$ (38). All of our data indicated that the *tox*-201 mutation affected regulation of toxinogenesis but did not alter the structure or activity of diphtherial toxin. Complementation analysis demonstrated that the *tox*-201 mutation affected expression of the toxin structural gene in a *cis*-dominant manner (Table 2). With C7($\beta^{tox\text{-}201}$) and wild-type C7(β), the highest differential rates of toxin production were observed only during the terminal stages of bacterial growth in batch cultures in low-iron medium (Fig. 3). When excess iron was present, the differential rate of toxin production decreased significantly with C7 ($\beta^{tox\text{-}201}$) but decreased almost to undetectable levels with wild-type C7(β). We conclude that toxin production by *C. diphtheriae* C7(β) is regulated in a complex manner that depends both on the availability of iron and on the stage of bacterial growth. The iron effect in *C. diphtheriae* C7(β) is controlled at least in part by the genetic locus defined by the *tox*-201 mutation in phage β. However, production of toxin by *C. diphtheriae* also has characteristics that resemble production of secondary metabolites in other bacterial systems (61).

Relatively little is known about the molecular mechanisms that regulate toxinogenesis in *C. diphtheriae*. The isolation of *tox* mutants of phage β that coded for nontoxic CRMs related to toxin (18, 26, 32, 55, 57, 58) and the demonstration that toxin or appropriate CRMs were synthesized in vitro in bacterial extracts programmed with wild-type or mutant β phage DNAs (37) established unequivocally that the genome of phage β contains the structural gene for toxin. Diphtherial toxin is produced as an extracellular protein (4, 7, 41, 48), and no substantial pool of intracellular toxin exists in toxin-producing cells of *C. diphtheriae* (4, 7, 41, 45). Radioisotopic tracer experiments showed that toxin is formed by *C. diphtheriae* in association with the cell membrane (60) and is secreted rapidly (59). Recent studies indicate that toxin is synthesized by membrane-associated poly-

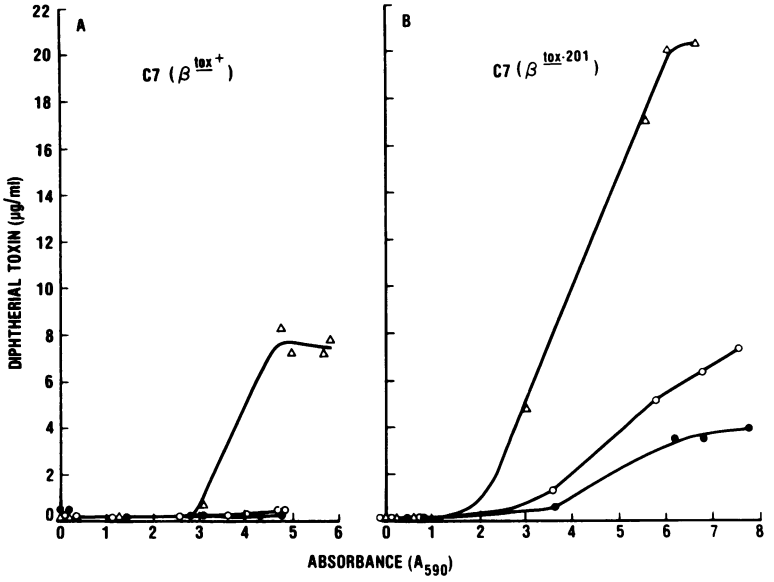

FIG. 3. *Differential rates of toxin production by C. diphtheriae C7(β^{tox+}) (A) and C7($\beta^{tox-201}$) (B) in cultures containing varying concentrations of added iron. Extracellular toxin was plotted as a function of bacterial growth (absorbance at 590 nm [A_{590}]). The concentrations of added Fe^{2+} were as follows: 0.075 μg/ml (△), 0.3 μg/ml (○), and 1.0 μg/ml (●). Experimental procedures were as described in the legend to Fig. 2 and in the text.*

TABLE 2. *Complementation tests: control of expression of the toxin structural gene by the tox-201 regulatory allele*

Expt	Lysogenic bacterial strain	Superinfecting phage	Iron concn[a]	Toxin concn (μg/ml)[b]	CRM26 concn (μg/ml)[b]
1	C7(β^{tox+})	None	Low	10	ND[c]
			High	0.05	ND
2	C7(β^{tox-2})	None	Low	<0.01[d]	1.1 ± 0.14
			High	<0.01	0.03 ± 0.01
		β^{tox+}	Low	9.5 ± 5.7	ND
			High	0.02 ± 0.01	ND
		$\beta^{tox-201}$	Low	17 ± 7.0	ND
			High	0.54 ± 0.22	ND
3	C7($\beta^{tox-201}$)	None	Low	32 ± 5.5	ND
			High	1.2 ± 0.42	ND
		β^{tox+}	Low	36 ± 0.71	ND
			High	1.2 ± 0.08	ND
		$\beta^{tox-201}$	Low	36 ± 4.2	ND
			High	1.2 ± 0.48	ND
4	C7($\beta^{tox-201\ tox-2}$)[e]	None	Low	<0.01	4.3 ± 1.9
			High	<0.01	0.57 ± 0.11
		β^{tox+}	Low	4.2 ± 1.2	ND
			High	0.02 ± 0.01	ND
		$\beta^{tox-201}$	Low	9.1 ± 2.7	ND
			High	0.23 ± 0.10	ND

[a] Low iron, 0.075 μg of added Fe^{2+} per ml; high iron, 1.0 μg of added Fe^{2+} per ml.
[b] Determined by radioimmunoassays as described in the text and reference 9. CRM26 was measured as equivalents of purified fragment A.
[c] ND, Not done.
[d] The smallest quantity of toxin or CRM antigen detectable by the radioimmunoassays was 0.01 μg/ml.
[e] The $\beta^{tox-201\ tox-2}$ prophage also contained the host range markers *h* and *h'*. See text.

somes as a larger precursor molecule that is cotranslationally secreted and proteolytically processed to extracellular toxin (51, 52).

mRNA extracted from toxin-producing cells of *C. diphtheriae* can be translated in vitro to produce toxin in appropriate extracts from *Escherichia coli* (28) or *C. diphtheriae* (24). When excess iron is added to toxin-synthesizing continuous cultures of *C. diphtheriae*, formation of extracellular toxin ceases abruptly (47). The kinetics of decay of toxin-specific mRNA in vivo, as measured by determinations of residual toxin-synthesizing capacity, are indistinguishable after the addition of iron or rifampin (36). These data support the hypothesis that regulation of transcription of toxin-specific mRNA is affected by iron. Factors in crude or partially purified extracts of *C. diphtheriae* have been reported to inhibit selectively both phage β DNA-directed (37) and mRNA-directed (24) in vitro synthesis of diphtherial toxin. These factors were not highly purified, nor have their mechanisms of action been clearly defined at the biochemical level.

Mutations have been identified in the genomes of phage β (38; see above) and *C. diphtheriae* C7 (23; Cryz and Holmes, manuscript in preparation) that confer complete or partial resistance to the iron effect. The *cis*-dominant expression of the β^{tox+}_{cI1} and $\beta^{tox\text{-}201}$ regulatory mutants (38; see above), the reported properties of the bacterial mutation in *C. diphtheriae* C7(β)hm723 (23), the presence of a toxin-specific repressor-like factor in extracts of *C. diphtheriae* (37, 38), and the rapid decay of toxin-synthesizing capacity after addition of iron to cultures of *C. diphtheriae* (36) are all consistent with the model of Murphy and his collaborators (35, 38) postulating a negatively regulated *tox* operon in phage β. Nevertheless, these data are not sufficient to prove the model or to exclude other possible models for regulation of diphtherial toxin.

The observations summarized above concerning the iron effect on toxinogenesis in *C. diphtheriae* should be compared with studies of metabolic regulation in other bacterial systems (21, 25, 33, 54). The most likely genetic elements to be considered as candidates for the *cis*-dominant *tox*-201 regulatory site are the promoter, the operator, and the attenuator. Each of these sites should be closely linked to the proximal end of the transcriptional unit which it regulates. Recent mapping experiments in our laboratory confirmed the prediction that the *tox*-201 mutation is contiguous to the *tox* locus of phage β and is closely linked to the toxin structural gene on the side corresponding to the amino-terminal end of diphtherial toxin (63).

The *tox*-201 regulatory mutation in phage β is associated with a complex phenotype. Strain C7($\beta^{tox\text{-}201}$) produced almost four times as much toxin as wild-type C7(β) under optimal low-iron conditions. Under high-iron conditions, the yield of toxin from C7($\beta^{tox\text{-}201}$) was almost 200 times greater than the yield from wild-type C7(β). Toxin was produced by C7($\beta^{tox\text{-}201}$) only during the terminal stages of bacterial growth. Excess iron decreased the differential rate of synthesis of toxin by C7($\beta^{tox\text{-}201}$), but this iron effect was much less dramatic than with wild-type C7(β). These phenotypic properties of the *tox*-201 mutation resemble some characteristics of both superpromoter (p^s) mutations (1, 9, 46, 49) and operator-constitutive (o^c) mutations (21, 33, 54), but attenuator mutations can also cause constitutive *cis*-dominant expression of the structural genes of an operon (22, 53). In *E. coli* abnormal species of tRNA that are incompletely modified are produced under conditions of iron starvation, both in vitro and in infected animals (14). The possibility that altered tRNA's may also be produced by *C. diphtheriae* under conditions of iron starvation and that they could influence expression of the toxin structural gene or other genes by mechanisms involving attenuation has not been excluded. Further studies will be needed to define at a molecular level the regulatory mechanism affected by the *tox*-201 mutation in phage β.

The role of excess iron provides one of the most striking examples of a specific metabolic factor that affects toxinogenesis in *C. diphtheriae*, and therefore the iron effect has received extensive study. However, it is also clear that the regulation of toxinogenesis is complex and that factors other than iron are important for toxinogenesis. In the present study, our results with the mutant lysogen C7($\beta^{tox\text{-}201}$) demonstrated that toxin was formed only during the terminal stages of bacterial growth even in high-iron medium in which iron was not the growth-limiting factor. Thus, some physiological change associated with the terminal stages of growth may represent a necessary factor for toxinogenesis that is distinct from iron deficiency. Mutants of phage β and of *C. diphtheriae* C7 that are altered in the regulation of toxinogenesis will be useful for continuing studies of the genetics, biochemistry, and molecular biology of toxinogenesis in *C. diphtheriae*.

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