

Regulation of Toxinogenesis in *Corynebacterium diphtheriae*: Mutations in the Bacterial Genome That Alter the Effects of Iron on Toxin Production

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Mutants of *Corynebacterium diphtheriae* C7(β) that are resistant to the inhibitory effects of iron on toxinogenesis were identified by their ability to form colonies surrounded by toxin-antitoxin halos on agar medium containing both antitoxin and a high concentration of iron. Chromosomal mutations were essential for the altered phenotypes of four independently isolated mutant strains. During growth in deferrated liquid medium containing various amounts of added iron, these mutants differed from wild-type *C. diphtheriae* C7(β) in several ways. Their growth rates were slower under low-iron conditions and were stimulated to various degrees under high-iron conditions. The concentrations of iron at which optimal toxin production occurred were higher for the mutants than for wild-type *C. diphtheriae* C7(β). Toxin production by the mutants during growth in low-iron medium occurred throughout the period of exponential growth at nearly constant rates that were proportional to the bacterial growth rates. In contrast, toxin production by wild-type *C. diphtheriae* C7(β) in similar low-iron cultures occurred predominantly during the late exponential phase, when iron was a growth-limiting nutrient. Additional studies demonstrated that these mutants had severe defects in their transport systems for ferric iron. We propose that the altered regulation of toxinogenesis by iron in our mutants was caused by the severe defects in their iron transport systems. As a consequence, the mutants exhibited a low-iron phenotype during growth under conditions that permitted wild-type *C. diphtheriae* C7(β) to exhibit a high-iron phenotype.

The structural gene for diphtheria toxin is present in different corynebacteriophages, including the well-studied phage β (4, 6, 14, 32). The production of diphtheria toxin by the lysogenic strain *Corynebacterium diphtheriae* C7(β) is affected by many factors, including the genotype of the bacterial host cell (2, 10, 19, 27, 29), the genotype of the β prophage (23, 33, 35), and the composition of the growth medium (20, 30). Among the nutritional factors that have been identified, the inhibitory effect of excess iron on toxinogenesis is particularly striking (3, 28). Maximal quantities of diphtheria toxin are produced by *C. diphtheriae* when cultures are grown in iron-deficient medium (3, 26). Mutations either in *C. diphtheriae* C7 (17) or in phage β (23, 35, 36) can alter the effects of iron on toxin production and enable mutant C7(β) lysogens to produce more toxin than wild-type C7(β) lysogens under high-iron conditions. Although the relationships between excess iron and decreased

toxin yields have been studied extensively, the precise mechanism(s) for the inhibitory effect of iron on toxinogenesis remains unknown (3, 22, 26, 28, 35).

Genetic analyses of regulatory mutations in phage β that affect control of toxinogenesis by iron have been reported. With the mutants designated β_{crl}^{tox+} (23) and $\beta^{tox-201}$ (35, 36), expression of the *tox* regulatory mutation was *cis* dominant. The *tox*-201 marker was mapped within the *tox* locus and shown to be contiguous to the origin of transcription of the toxin structural gene (36). The *tox*-201 mutation defines a regulatory site that could represent an operator, promoter, or attenuator involved in the inhibition of toxinogenesis by iron.

Kanei et al. described several mutants of C7(β) that produced high yields of diphtheria toxin under high-iron conditions (17). In one strain, designated C7hm723, the mutation controlling the altered phenotype was shown to reside in the host cell genome. Growth of C7hm723 was indistinguishable from growth of wild-type C7(β), but the mutant lysogen pro-

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duced as much toxin under high-iron conditions as the wild-type C7(β) lysogen produced under optimal low-iron conditions. These observations were consistent with the hypothesis that C7hm723 fails to produce a bacterial regulatory factor postulated to be essential for the inhibition of toxin production by iron (17, 22, 23). The possibility that C7hm723 might be defective in iron transport was recognized but could not be established or excluded by the experimental data (17).

In this communication we report the isolation and preliminary characterization of additional mutants of *C. diphtheriae* C7(β) that produced large amounts of toxin during growth in high-iron medium. The properties of our mutants varied from strain to strain and also differed from those reported for C7hm723 (17). Chromosomal mutations in our newly isolated strains were essential for their altered phenotypes. We demonstrated that all of our mutants were severely defective in their ability to transport ferric iron, and C7hm723 was shown to be moderately defective in iron transport. This collection of bacterial mutants provides an excellent resource for continuing genetic and biochemical studies of the regulation of toxinogenesis and its relationship to iron metabolism in *C. diphtheriae*.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The properties of *C. diphtheriae* C7 and C7(β) have been previously described (12, 13). *C. diphtheriae* C7hm723 was provided by T. Uchida, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan (17). PGT medium was the casein hydrolysate medium of Mueller and Miller (20) modified as described by Barksdale and Pappenheimer (7). Maltose was added to the medium to a final concentration of 20 g/liter. PGT-maltose medium was deferrated in accordance with published procedures (20), and supplemental FeSO₄ or FeCl₃ was added for specific experiments in the amounts indicated in the text. Methods for the cultivation of bacterial strains, the propagation and assay of bacteriophages, the construction of lysogenic strains, and the production of diphtheria toxin in low-iron PGT-maltose medium have been described previously (12, 13, 35, 36). Culture supernatants to be assayed for diphtheria toxin by radioimmunoassay were collected by centrifugation, sterilized by filtration through membrane filters (type GA; pore size, 0.45 μ m; Gelman Sciences Inc., Ann Arbor, Mich.), stored at 4°C, and tested within 24 h (12, 35). Bacterial growth was determined by measuring the absorbance at 590 nm (A_{590}).

Diphtheria toxin, toxoid, and antitoxin. Purified diphtheria toxins Jn141 and Dc202 (8, 15), hyperimmune goat antiserum raised against Dc202 toxoid (150 antitoxin units per ml) (34), and hyperimmune rabbit antiserum R21 raised against Jn141 toxoid (600 antitoxin units per ml) (8) were used.

Tests for diphtheria toxin. (i) Antitoxin-agar plate

assay. We used antitoxin-agar containing 10 μ g of Fe²⁺ per ml to screen mutagenized cultures of *C. diphtheriae* C7(β) for rare colonies that produced toxin under high-iron conditions (17, 25). Control experiments demonstrated that concentrations of iron of 1 μ g/ml or greater were sufficient to inhibit the formation of toxin-antitoxin halos around colonies of wild-type C7(β). In each plastic petri dish (15 by 100 mm) we mixed 10 ml of modified M-4 agar (16) cooled to 50°C, 2 ml of heat-inactivated horse serum (GIBCO Laboratories, Grand Island, N.Y.), 24 units of goat antitoxin, and 120 μ g of Fe²⁺ as FeSO₄. Plates were inoculated with mutagenized *C. diphtheriae* to yield approximately 200 colonies per plate, incubated for 48 h at 32°C, and examined for colonies surrounded by toxin-antitoxin halos.

(ii) **Elek test.** Qualitative tests for the production of toxin antigen by *C. diphtheriae* strains were performed by a modification (12) of the method of Elek (9). For certain experiments, the medium was supplemented with various concentrations of FeSO₄, as indicated in the text.

(iii) **Radioimmunoassay.** A quantitative, competitive-binding radioimmunoassay for toxin antigen present in supernatants from cultures of *C. diphtheriae* was performed as described elsewhere (8).

(iv) **Toxicity tests.** The toxicity of sterile supernatants from cultures of various strains of *C. diphtheriae* was determined in rabbits by intracutaneous tests (5).

Chemical mutagenesis. The chemicals *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) and ethyl methane sulfonic acid ester (EMS) (both from Sigma Chemical Co., St. Louis, Mo.) were used to induce mutations in *C. diphtheriae* C7(β). Single-colony isolates of C7(β) were inoculated into 10-ml samples of PGT-maltose medium and grown to the mid-log phase (A_{590} , 0.3). The cells were then harvested by centrifugation at 12,000 $\times g$ for 15 min at 4°C. For mutagenesis by NG (1) the cells were washed twice with 10 ml of 50 mM Tris-50 mM maleate buffer (pH 6.0) and resuspended in 0.5 ml of the same buffer. NG was added to yield a final concentration of 670 μ g/ml, and the cell suspension was incubated for 15 min at 34°C without shaking. The treated cells were washed twice with 10 ml of Tris-maleate buffer, suspended in 10 ml of PGT-maltose medium, and incubated for 16 h at 37°C before being screened for mutants by the antitoxin-agar plate assay. Viable counts performed immediately before and after treatment with NG indicated that approximately 99% of the cells were killed. For mutagenesis by EMS (18) logarithmic-phase C7(β) cells were washed twice in 10 ml of 0.1 M Tris-hydrochloride buffer (pH 7.5) and resuspended in the same buffer to a final volume of 5.0 ml, and 0.075 ml of undiluted EMS was added. The suspension was incubated at 34°C for 2 h with occasional gentle mixing. Cells were then washed twice with 10 ml of Tris-hydrochloride buffer, inoculated into 10 ml of PGT-maltose medium, and incubated at 37°C for 16 h before being screened for mutants. EMS treatment caused an approximately 90% decrease in cell viability.

Iron uptake studies. Cells to be used for transport studies were grown to the log phase (A_{590} , 0.5) in 10-ml samples of deferrated PGT-maltose medium with 0.075 μ g of added Fe³⁺ per ml in 125-ml disposable Nalgene flasks (Nalge Co., Rochester, N.Y.) in a water bath incubator at 37°C with rotary shaking. Uptake was

TABLE 1. Effects of iron on the production of diphtheria toxin by wild-type and mutant strains of *C. diphtheriae* C7(β) in 18-h cultures

Strain	Extracellular toxin produced ($\mu\text{g/ml}$) at indicated concentration ^a of added iron								
	0	0.05	0.075	0.1	0.2	0.3	1.0	3.0	10.0
C7(β)	2.04	2.18	2.54	2.20	0.36	0.14	0.09	0.72	0.04
Mutant HC1	0.06	2.20	4.40	6.20	5.40	2.28	1.60	1.40	0.73
Mutant HC3	0.02	0.72	1.12	1.24	1.88	1.52	1.48	0.68	0.64
Mutant HC4	0.05	0.65	1.06	2.08	2.71	3.72	5.16	2.28	1.14
Mutant HC5	0.04	0.76	0.92	1.60	3.20	3.88	3.60	3.20	6.10 ^b
C7hm723	4.30	4.32	3.62	3.85	3.41	2.13	3.27	1.99	1.87

^a In micrograms of Fe^{2+} per milliliter.

^b The high concentration of toxin in this sample was not typical. In another experiment, supernatants from cultures of HC5 contained 3.42 μg of toxin per ml when the concentration of added iron was 3.0 $\mu\text{g/ml}$ and 2.02 μg of toxin per ml when the concentration of added iron was 10.0 $\mu\text{g/ml}$.

initiated by the addition of 1 μg of $^{59}\text{Fe}^{3+}$ (as $^{59}\text{FeCl}_3$ in 100 μl of 0.1 M HCl; specific activity, 10 $\mu\text{Ci}/\mu\text{g}$; Amersham Corp., Arlington Heights, Ill.). Samples (0.5 ml) were withdrawn from the cultures at appropriate times, and the bacteria were collected by filtration on nitrocellulose filters (pore size, 0.45 μm ; Schleicher & Schuell Co., Keene, N.H.). The filters were washed twice with 1.0-ml volumes of Dulbecco phosphate-buffered saline without CaCl_2 and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (pH 7.2) (GIBCO Laboratories), and counted in a Searle 1185 gamma counter. Nonspecific adsorption of radioactivity to the filters under these conditions was insignificant (ca. 30 cpm per sample). The amount of iron taken up was expressed as nanograms of $^{59}\text{Fe}^{3+}$ per sample (containing ca. 1.7×10^8 cells).

RESULTS

Isolation of mutants. Cultures of *C. diphtheriae* C7(β) were treated with NG or EMS, and the mutagenized bacteria were plated on antitoxin-agar plates to screen for mutants that produced diphtheria toxin under high-iron conditions. Four independently derived mutants were detected among approximately 488,000 colonies examined. Mutants HC1, HC4, and HC5 were from cultures treated with NG (mutant frequency, ca. 4×10^{-5}), and mutant HC3 was obtained from cultures treated with EMS (mutant frequency, ca. 5×10^{-5}). All four mutants produced toxin that was antigenically indistinguishable from purified diphtheria toxin in competitive-binding radioimmunoassays and was highly toxic for rabbits in intracutaneous tests (data not shown). Thus, the mutations in our newly isolated strains altered the regulation of toxinogenesis by iron but did not cause detectable changes in the structure or activity of diphtheria toxin.

Mutations affecting the ability of *C. diphtheriae* C7(β) to produce toxin under high-iron conditions might occur in the β prophage, in the bacterial genome, or in both. The β phages carried by each of the four mutant lysogens and by the C7hm723 lysogen (17) were isolated and used to construct new C7 lysogens. In vitro tests

for toxin production by the parental strains and the newly constructed lysogens were performed by the Elek method in media containing 0, 1, or 10 μg of added iron per ml. Mutants HC1, HC3, HC4, HC5, and C7hm723 produced detectable toxin antigen at all of these iron concentrations. In contrast, wild-type C7(β) and all of the newly constructed lysogens produced toxin only in media containing no added iron. These observations confirmed reported findings with C7hm723 (17) and also established that mutations in the bacterial genome were essential for mutants HC1, HC3, HC4, and HC5 to synthesize diphtheria toxin under high-iron conditions. Repeated efforts to cure mutants HC1, HC3, HC4, and HC5 of their resident prophages were not successful.

Toxin production by mutants. To obtain quantitative data concerning the effects of iron on toxinogenesis, we grew each bacterial strain in deferrated PGT-maltose medium containing from 0 to 10 μg of added iron per ml and determined the final yield of extracellular toxin by radioimmunoassay (Table 1). Maximal toxin yields with wild-type C7(β) were achieved when the concentration of added iron was approximately 0.075 $\mu\text{g/ml}$, confirming published observations (35, 37). With initial iron concentrations of 0.2 $\mu\text{g/ml}$ or greater, toxin production by wild-type C7(β) decreased to low levels. With our newly isolated mutants HC1, HC3, HC4, and HC5 the concentrations of iron required for optimal toxin yields were higher, and the ranges of iron concentrations over which large amounts of toxin could be produced were broader than with wild-type C7(β). The poor yields of toxin in media containing no added iron correlated with the poor growth of our mutants under these conditions (data not shown). In contrast, C7hm723 produced substantial amounts of toxin at all concentrations of iron tested.

We next examined bacterial growth and toxin production as a function of time during cultivation in deferrated PGT-maltose medium with

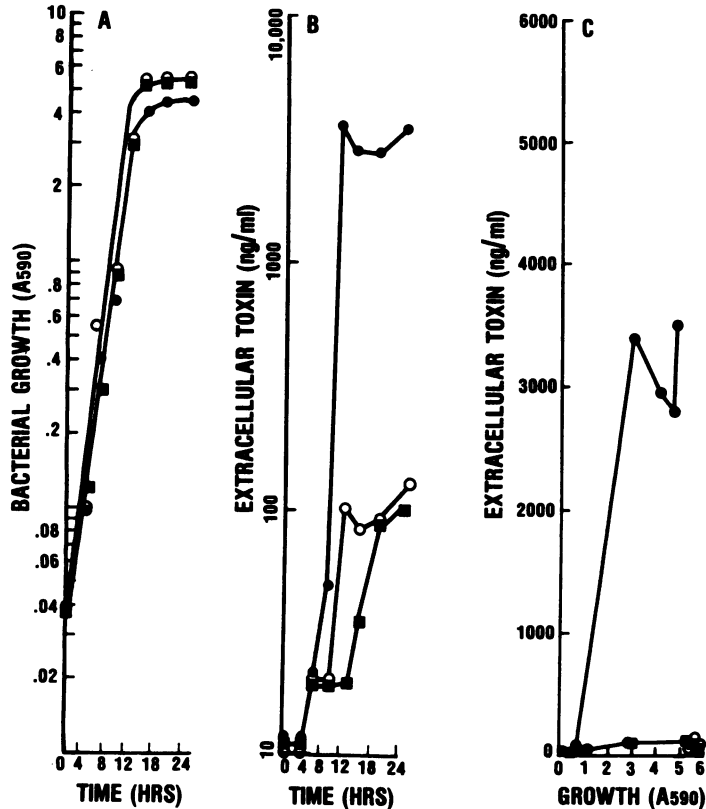


FIG. 1. Growth and toxin production by *C. diphtheriae* C7(β) in PGT-maltose medium with various concentrations of added iron. Symbols: ●, 0.075 μg of Fe^{2+} per ml; ■, 0.3 μg of Fe^{2+} per ml; ○, 1.0 μg of Fe^{2+} per ml.

various concentrations of added iron. Representative data for strains C7(β), HC3, and HC5 are shown in Fig. 1, 2, and 3, respectively. Four characteristics of wild-type C7(β) grown under these conditions are noteworthy. The growth rates were similar in low-iron and high-iron media, the final growth yield was lower in low-iron medium than in high-iron medium, toxin production occurred predominantly during the late exponential phase of growth, and toxin production was minimal in medium containing 0.3 or 1.0 μg of iron per ml (Fig. 1). Among our mutant strains, HC3 was most different from wild-type C7(β) (Fig. 2). The growth rate and final growth yield of HC3 were less than those of C7(β) and were not significantly affected by changes in the concentration of iron in the medium within the range of concentrations tested. Intermediate results were obtained with HC5 (Fig. 3) and with HC1 and HC4 (data not shown). As the concentration of iron in the

medium increased, the growth of HC1, HC4, or HC5 was stimulated, and toxin production decreased. We confirmed the observations of Kanei et al. (17) that C7hm723 resembled C7(β) in its growth characteristics but produced toxin continuously in amounts approximately proportional to the amount of bacterial growth at all concentrations of iron tested (data not shown).

Iron uptake by mutants. The uptake of $^{59}\text{Fe}^{3+}$ at 37°C by logarithmic-phase cells of C7(β) grown under low-iron conditions was linear for about 20 min and reached a maximum of approximately 14 ng per sample by 30 min (Fig. 4a). A similar uptake curve was obtained for the isogenic, nonlysogenic strain *C. diphtheriae* C7, demonstrating that iron uptake is determined by the bacterial host and not by prophage β (data not shown). The uptake of iron was reduced almost to background levels at 4°C. Additional studies characterizing the active transport system for ferric ions in *C. diphtheriae* will be reported elsewhere (L. M. Russell and R. K. Holmes, manuscript in preparation). Mutants HC1, HC3, HC4, and HC5 all showed an ex-

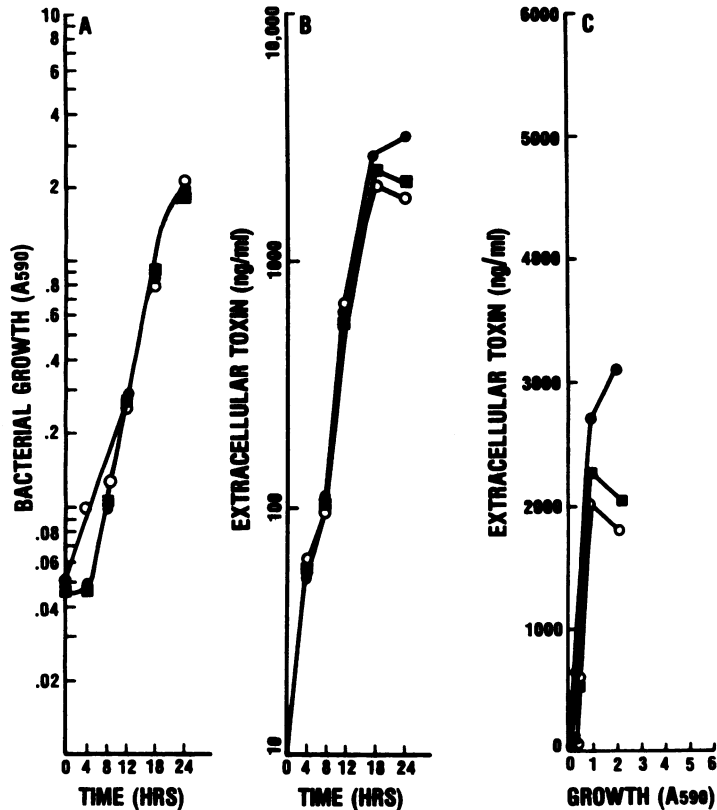


FIG. 2. Growth and toxin production by *C. diphtheriae* mutant HC3 in PGT-maltose medium with various concentrations of added iron. See legend to Fig. 1 for explanation of symbols.

tremely limited ability to take up iron; in contrast, mutant C7hm723 transported iron at a rate about half that of wild-type cells (Fig. 4b).

DISCUSSION

In the present study we isolated and characterized four chromosomal mutants of *C. diphtheriae* C7(β) able to produce large amounts of diphtheria toxin under high-iron conditions. These mutants differed from wild-type *C. diphtheriae* C7(β) in several phenotypic properties. The differences included slower growth rates under low-iron conditions, stimulation of growth rates toward wild-type levels under high-iron conditions, optimal toxin production at higher concentrations of iron, and the ability to produce toxin continuously throughout the exponential phase of growth in low-iron medium. All of our newly isolated mutants were extremely defective in their abilities to transport iron. Although differences among these strains were demonstrated, their phenotypes were very similar. It is possible that differences in the residual iron uptake activities of these mutants which

were not demonstrated under the assay conditions used might explain the slight differences in their phenotypes.

Because many bacterial functions require iron, defects in iron transport may affect multiple biochemical pathways (24). It seems likely that the defects in iron transport in mutants HC1, HC3, HC4, and HC5 were severe enough to affect a variety of processes that are dependent on intracellular iron. Although we cannot yet exclude the possibility of multiple mutations in our mutants, we favor the hypothesis that the primary defects in these strains involve components of their iron transport systems. As a result of the deficiencies in iron transport, the phenotypes of the mutants would resemble the low-iron phenotype of C7(β), even during growth under high-iron conditions. The resistance of mutants HC1, HC3, HC4, and HC5 to the inhibitory effects of iron on toxin production, which was the basis for their initial isolation, would thus be one of several secondary consequences of defective iron transport.

The phenotypes of the mutants reported here are significantly different from that of *C. diph-*

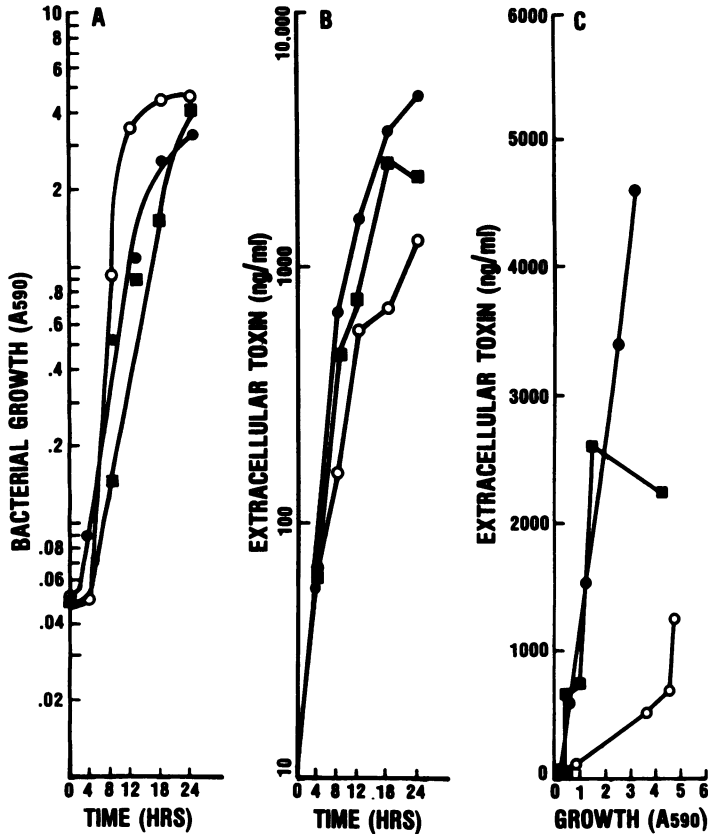


FIG. 3. Growth and toxin production by *C. diphtheriae* mutant HC5 in PGT-maltose medium with various concentrations of added iron. See legend to Fig. 1 for explanation of symbols.

theriae C7hm723 (17). Although we demonstrated a decreased rate of iron uptake by C7hm723, the defect in iron transport was less dramatic than for our mutants, and growth of C7hm723 was indistinguishable from that of wild-type C7(β). If defective iron uptake is responsible for the resistance of C7hm723 to the inhibitory effects of iron on toxinogenesis, then it is necessary to postulate that toxin production can occur at a maximal rate during an early stage of iron depletion and before the growth rate decreases. Based on experiments with a different strain of *C. diphtheriae*, Hirai et al. (11) also postulated that different amounts of iron were needed to inhibit toxinogenesis and to permit maximal rates of growth. A second possibility is that a single gene product which is altered by the mutation in C7hm723 has functions that are involved both in iron transport and in the regulation of toxinogenesis. We cannot exclude the possibility that C7hm723 has multiple mutations that affect iron uptake and the regulation of toxinogenesis by independent mechanisms.

Recent work with *Pseudomonas aeruginosa*

led to the identification of mutants that were resistant to the inhibitory effects of iron on the production of a group of extracellular products, including exotoxin A, elastase, alkaline protease, hemagglutinin, pyochelin, and pyoverdine (31). Mutants of this class were shown to have defects in iron transport. Mutants of *P. aeruginosa* of another type made large amounts of a specific product such as exotoxin A or elastase under high-iron conditions, but the production of other iron-regulated extracellular products was suppressed in the usual manner (31). Thus, mutations that alter the effects of iron on toxinogenesis in *P. aeruginosa* can affect either iron transport or specific regulatory functions that are dependent on iron.

The studies reported here have demonstrated that alterations in the regulation of toxinogenesis by iron in four independently isolated mutants of *C. diphtheriae* are associated with severe defects in iron transport. These mutants will be useful for future studies of the molecular mechanisms linking iron transport with the regulation of toxinogenesis in *C. diphtheriae*.

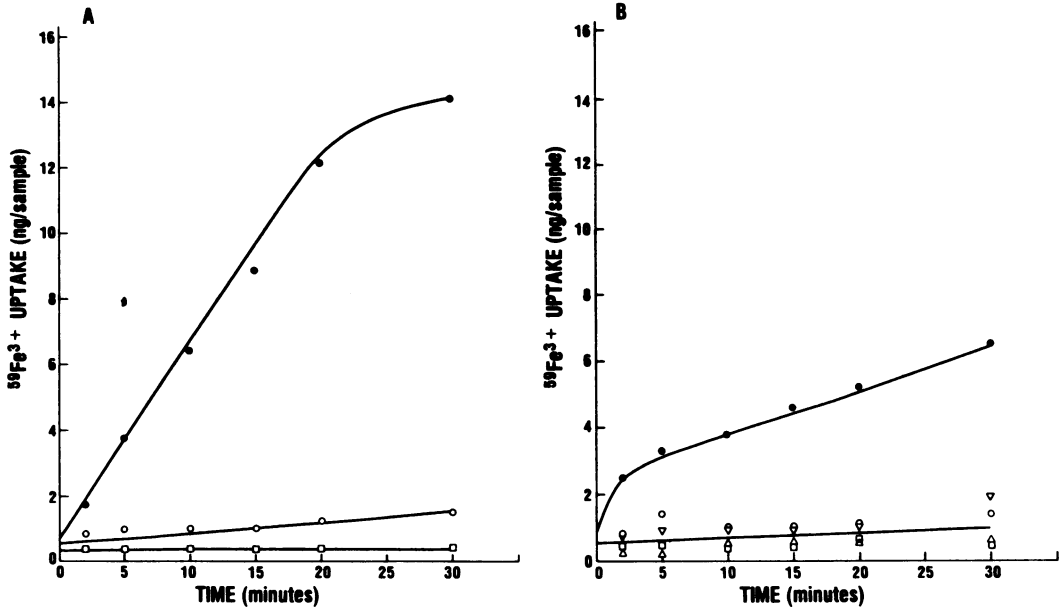


FIG. 4. Uptake of $^{59}\text{Fe}^{3+}$ by wild-type and mutant *C. diphtheriae* strains. Uptake experiments were performed as described in the text with cells grown in medium containing $0.075\ \mu\text{g}$ of added Fe^{3+} per ml. (A) Uptake of $^{59}\text{Fe}^{3+}$ by wild-type C7(β) cells at 37°C (●) or 4°C (○); (□) uninoculated medium control at 37°C . (B) Uptake of $^{59}\text{Fe}^{3+}$ by mutants HC1 (○), HC3 (□), HC4 (△), HC5 (▽), and C7hm723 (●). The least-squares regression lines for the data for mutants HC1, HC3, HC4, and HC5 did not differ significantly, and a single regression line based on the pooled data for these strains is shown.

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