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Genetic and Biochemical Evidence for a Siderophore-Dependent Iron Transport System in *Corynebacterium diphtheriae*

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During growth under conditions of iron deprivation, *Corynebacterium diphtheriae* secreted a siderophore into the culture medium. This extracellular siderophore was necessary for maximal rates of iron uptake at pH 8.0 by *C. diphtheriae* C7 and related strains. We isolated a mutant of *C. diphtheriae* C7(β), strain HC6, which did not make the corynebacterial siderophore. Strain HC6 grew very poorly, even under high-iron conditions, and had a severe defect in iron transport. Both growth and iron uptake by strain HC6 were greatly stimulated by the corynebacterial siderophore. We used strain HC6 to develop a bioassay for the corynebacterial siderophore and to look for other potential siderophores for *C. diphtheriae*. Among the purified phenolate and hydroxamate siderophores tested, only aerobactin was able to stimulate the growth of strain HC6. Partial purification of the corynebacterial siderophore was achieved. The siderophore did not give positive reactions in the Arnow test for phenolates or the Csaky test for hydroxamates and may have a novel chemical structure.

Iron uptake into many pathogenic bacterial strains is facilitated by extracellular siderophores which compete for iron with host iron-binding proteins. The siderophores present the chelated iron to specific bacterial transport systems (14, 16, 20). Most siderophores which have been identified belong to one of two chemical classes: they are either phenolates or hydroxamates (20). The production of these factors is genetically determined and regulated by the iron concentration of the medium (8, 19). The ability of pathogenic bacteria to produce siderophores is often correlated with virulence (8, 16, 30).

In *Corynebacterium diphtheriae* iron is important for many different biochemical pathways. The production of diphtheria toxin by *C. diphtheriae* is inhibited by high concentrations of iron in the growth medium (2, 21). The morphology of the cell envelope and synthesis of components of the electron transport chain and coproporphyrin III also reflect the extracellular iron concentration (reviewed in reference 2). The production of coproporphyrin III by *C. diphtheriae* and its excretion into the medium parallel that of diphtheria toxin (5, 33). Several models have been proposed to explain the way in which synthesis of diphtheria toxin is regulated by iron (2, 17, 22), but direct biochemical confirmation of the actual regulatory mechanism(s) is lacking.

Because iron has many physiological roles in *C. diphtheriae*, an understanding of the means by which iron is transported into *C. diphtheriae* is important. Recent studies in our laboratory characterized iron uptake into *C. diphtheriae* as an active transport process specific for the ferric ion and provided preliminary evidence supporting the involvement of a corynebacterial siderophore in iron uptake (28). We demonstrated the presence of significant amounts of an iron-solubilizing activity in supernatants from cultures of *C. diphtheriae* grown under low-iron conditions (13) and showed that a dialyzable factor present in such low-iron culture supernatants was necessary for maximal rates of iron uptake at pH 8.0 (28). In this paper we provide additional evidence for a corynebacterial siderophore, including the isolation of a siderophore-deficient mutant of *C. diphtheriae*, the development of specific bioassays to detect siderophore activity, and partial purification of the siderophore. The corynebacterial siderophore is different from a number of previously characterized phenolate and hydroxamate siderophores and may have a novel chemical structure.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacterial strains were from our stock culture collection and were kept frozen at −70°C for long-term maintenance. The toxinogenic *C. diphtheriae* strain C7(β), the isogenic but nonlysogenic strain C7, and the nontoxinogenic strain C7(γ) have all been previously described (3, 11). *C. diphtheriae* strains C7($\beta^{tox\text{-}201}$), C7($\beta^{tox\text{-}202}$), and C7($\beta^{tox\text{-}203}$) were isolated in our laboratory (32), and strain C7(β^{tox+}_{cr1}) was obtained from J. R. Murphy, Harvard Medical School (18). The *C. diphtheriae* mutant strains HC1, HC3, HC4, and HC5 were isolated in our laboratory and reported previously (6). Lysogenic derivatives of C7 were constucted by published methods (12). *Salmonella typhimurium enb*-7 was obtained from J. B. Neilands, University of California, Berkeley, and maintained on tryptose agar (TA) plates (12) at 37°C. *Arthrobacter flavescens* JG-9 was obtained from R. A. Finkelstein, University of Missouri, and maintained on Colombia blood agar plates (BBL, Microbiology Systems, Cockeysville, Md.) at 32°C. *Shigella flexneri* M4243 was obtained from A. D. O'Brien, Uniformed Services University of the Health Sciences, and maintained on TA plates at 37°C.

C. diphtheriae strains were grown at 37°C on solid media on either TA or, when low-iron conditions were required, on DA-1 agar (31). Cultures in liquid medium were grown from small inocula (absorbance at 590 nm, 0.05) with shaking at 37°C in deferrated PGT-maltose medium (normally 10-ml volumes) to which iron was usually added at a concentration of either 0.075 μg of Fe^{3+} per ml (defined as low-iron conditions) or 1.0 μg of Fe^{3+} per ml (defined as high-iron conditions) (3). For some experiments bacteria were grown in deferrated PGT-maltose medium with no added iron. Conditioned medium refers to supernatants from cultures of

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C. diphtheriae C7(β) grown for 20 h (to stationary phase) under either low-iron or high-iron conditions. Cells were removed by centrifugation for 10 min at 12,000 × *g*, and the supernatants were kept at 4°C.

Chemicals. All chemicals used were of the highest grade commercially available. Nitrilotriacetic acid (NTA) was purchased from the Sigma Chemical Co., St. Louis, Mo., as the disodium salt. Stock solutions of 10 mM NTA were made up in quartz-distilled water and stored at 4°C. Ethylenediamine-di(*o*-hydroxyphenyl acetic acid) (EDDA; 90% purity) was purchased from Sigma and further purified by the procedure of Rogers (27). Stock solutions of 100 mg/ml were made by dissolving the EDDA in 1.0 M NaOH, bringing the pH to 7.4 with HCl, and making the solution up to volume with quartz-distilled water. When needed, EDDA was added to the autoclaved DA-1 medium to a final concentration of 40 μg/ml. 2,3-Dihydroxybenzoic acid (DHBA) was purchased from Sigma. Purified enterochelin was a gift from H. Rosenberg, Australian National University, and purified vibriobactin and aerobactin were gifts from S. Payne, University of Texas at Austin. Purified hydroxamates, including rhodotorulic acid, ferrichrome A, desferrioxamine B mesylate (Desferal), and a number of synthetic derivatives of hydroxamic acid, were obtained from R. Grady, Rockefeller University. These iron-chelating compounds were dissolved in minimum amounts of absolute ethanol and made up to the desired volumes with quartz-distilled water. Radioactive iron was obtained from Amersham Corp., Arlington Heights, Ill., as the chloride salt of $^{59}Fe^{3+}$ in 0.1 M HCl (specific activity, 10 μCi/μg).

Detection of extracellular iron-solubilizing activity. A modified version of the dialysis method of Macham and Ratledge (15) was used to test culture supernatants for their ability to solubilize ferric iron. Culture supernatants or other samples to be tested were adjusted to pH 8.0 with 0.1 M NaOH. One-milliliter samples were placed inside dialysis sacs (molecular weight exclusion limit, ca. 4,500), and 70-μl volumes containing 200 μg of Fe^{3+} and 5 μCi of $^{59}Fe^{3+}$ (as $FeCl_3$ in 0.1 M HCl) were added. The samples were dialyzed to equilibrium for 18 h against 10-ml volumes of 0.05 M Tris-hydrochloride buffer at pH 8.0 in 15-ml plastic centrifuge tubes agitated in a roller drum. The volumes of the samples did not change significantly during dialysis. Samples (200 μl) were withdrawn from the dialysate and counted in a Searle 1185 gamma counter. The amount of iron solubilized was calculated from the experimental data with the assumption that the distribution of total Fe^{3+} was the same as the distribution of $^{59}Fe^{3+}$. The iron-solubilizing capacity of the uninoculated PGT-maltose medium was about 2 μg of Fe^{3+} per ml and was subtracted as background from the experimental data. The Tris-hydrochloride buffer at pH 8.0 had no measurable iron-solubilizing activity.

Iron uptake studies. The preparation of cells and measurements of the uptake of $^{59}Fe^{3+}$ in exponentially growing cultures of *C. diphtheriae* strains have been described previously (6). When washed cell suspensions were required, samples of log-phase cells were centrifuged at 10,000 × *g* for 10 min, resuspended in the specific medium for each experiment, centrifuged again, and finally resuspended at an absorption of 0.5 at 590 nm (about 3.3×10^8 cells per ml) in the same medium. If necessary, NaOH was used to adjust the pH of the medium to 8.0. Washed cell suspensions were allowed to equilibrate in the shaking water bath at 37°C for 5 min before transport studies were initiated. All additions of compounds to be tested for their effects on iron transport were made 5 s before the addition of $^{59}Fe^{3+}$.

Isolation of a siderophore-deficient mutant of *C. diphtheriae*. Bacteria from log-phase cultures of C7(β) were collected and treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma) under nongrowing conditions for 15 min as described previously (6). Approximately 2.6% of the treated cells remained viable. The mutagenized cultures were diluted and plated on DA-1 medium to yield approximately 200 colonies per plate, and the plates were then incubated at 37°C. Plates were examined at 24 and 48 h, and all colonies were marked. Plates were reexamined at 72 h to detect small colonies that developed late. Among approximately 24,000 colonies surveyed, 414 were visible only after day 3 of incubation. These slow-growing colonies were picked to TA plates. From 258 isolates that formed colonies on TA, inocula were subcultured both to DA-1 plates and to DA-1 plates supplemented with 1 μg of added Fe^{3+} per ml, and the plates were incubated at 37°C for 24 h. Four strains were identified that displayed minimal growth on unsupplemented DA-1 plates but formed small colonies on the DA-1 agar supplemented with iron. One of these strains, designated HC6, was selected for further study.

Bioassays for siderophores. *C. diphtheriae* HC6 was used as the indicator strain for the corynebacterial siderophore. *S. typhimurium enb-7* was used as the indicator strain for phenolate-type siderophores, with enterochelin (50 μg per well) as a positive control. *A. flavescens* JG-9 was used as the indicator strain for hydroxamate-type siderophores, with cholyhydroxamic acid (50 μg per well) as a positive control. *S. flexneri* M4243 was used as an indicator known to respond to aerobactin and to enterochelin (24), with aerobactin (15 μg per well) as a positive control. Cells of the required strain were scraped off TA plates in the case of *C. diphtheriae* HC6, *S. typhimurium enb-7*, or *S. flexneri* M4243 or blood agar plates in the case of *A. flavescens* JG9 and resuspended in deferrated PGT medium at a density of about 2×10^8 cells per ml. Samples (0.1 ml) of the bacteria were spread on the surface of DA-1 plates or DA-1 plates containing EDDA (40 μg/ml). Plates were incubated at 37°C (for *C. diphtheriae* HC6, *S. typhimurium enb-7*, or *S. flexneri* M4243) or at 32°C (for *A. flavescens* JG9). After 48 h on DA-1 plates there was very little growth of *S. typhimurium enb-7* or *C. diphtheriae* HC6 and no visible growth of *A. flavescens* JG9. There was no visible growth of these strains or *S. flexneri* M4243 on DA-1 plus EDDA plates after the same period of incubation. Samples to be tested for siderophore activity were diluted as appropriate in deferrated PGT medium or sterile quartz-distilled water, and samples up to 50 μl in volume were added to wells punched in plates that had previously been inoculated with the appropriate indicator strain. The diameters of the zones of visible bacterial growth around each well were measured after 48 h of incubation.

Chemical assays for siderophores. The Arnow assay was used to detect the presence of compounds with phenolate groups (1). DHBA was used as a standard, and the red color produced was measured at 515 nm. The minimum detectable amount of diphenol in this procedure is 5 nmol (4). The presence of hydroxamates was determined by the Csaky procedure, using both unhydrolyzed and hydrolyzed samples (7, 9). This assay can detect 0.2 μg of hydroxylamine nitrogen (approximately 14 nmol of hydroxamate) (7). Hydroxylamine hydrochloride (Sigma) and cholyhydroxamic acid were used as standards, and the absorbance was measured at 526 nm. Coproporphyrin III was identified by its absorption spectrum at pH 1 with the characteristic Soret band at 400 nm (26). All spectrophotometric measurements were made on a Gilford 2400-2 spectrophotometer.

TLC. Ascending thin-layer chromatography (TLC) was performed at room temperature on precoated silica gel plates (250-μm thick, without fluorescent indicator; E. M. Science, Cincinnati, Ohio) using EtOH(reagent grade, 95%)–1 mM ammonium acetate buffer, pH 7.2 (80:20) as solvent. The plates were dried, and spots were visualized under longwave UV (366 nm) or by spraying with phenolate reagent (1 volume of 0.1 M $FeCl_3$ in 0.1 M HCl added to 1 volume of 0.1 M potassium ferricyanide solution) (29).

Partial purification of corynebacterial siderophore. Samples containing 100 ml of conditioned medium or uninoculated medium were lyophilized, and 75-ml volumes of methanol (high-pressure liquid chromatography [HPLC] grade) were added. After 24 h at room temperature, the insoluble matter was removed by filtration, and the extracts were concentrated by rotary evaporation to about 10 ml. Fifty-milliliter volumes of ethyl acetate were added, and the precipitates which formed were extracted into two 30-ml volumes of quartz-distilled water by shaking in separatory funnels. The aqueous extracts were collected, and the contaminating organic solvents were removed on the rotary evaporator. The extracts were lyophilized, and the residues were dissolved in 5-ml samples of quartz-distilled water to yield dark yellow solutions. Samples were stored at 4°C, and the siderophore activity was apparently stable over a period of several weeks. These preparations are referred to as concentrated siderophore.

Further purification of the concentrated siderophore was achieved by chromatography on silica columns followed by HPLC. Glass columns (0.9 by 25 cm) were packed with silica gel slurry (Research Specialties Co., Richmond, Calif.) in EtOH–1 mM ammonium buffer, pH 7.2 (80:20). Samples containing 0.5 ml of the concentrated siderophore preparation were applied and eluted with the EtOH-ammonium acetate solvent system. Samples (1.0 ml) were collected, and absorbance at 360 nm was monitored. Each sample was tested for ability to stimulate the growth of HC6 on DA-1 plates and for reactivity with phenolate reagent and fluorescence under UV light on silica plates. Fractions with siderophore activity were combined, the EtOH was taken off by rotary evaporation, and the samples were lyophilized. The residues were resuspended in 1.0-ml volumes of quartz-distilled water. Filtrates of these samples (250 μl) were applied to a reverse-phase high-pressure column (μBondapak C18; length, 30 cm; inner diameter, 3.9 mm; Waters Associates, Milford, Mass.). A Waters 6000A solvent delivery system was used to construct 0 to 50% acetonitrile gradients in water for the elution of the corynebacterial siderophore activity. The eluates were monitored at 360 nm with a Waters 450 variable wavelength detector. Peak samples were pooled, run briefly on the rotary evaporator, and lyophilized. The residues were dissolved in 0.5 to 1.0 ml of quartz-distilled water.

Extraction of coproporphyrin. Coproporphyrin III was separated from culture supernatants and from partially purified siderophore preparations by adsorption onto activated charcoal or $Mg(OH)_2$ gel (10). The coproporphyrin was released from the $Mg(OH)_2$ by dissolving the gel with glacial acetic acid. The pH was then adjusted to less than 1 by the addition of 2 M HCl. The red coproporphyrin was extracted into an equal volume of ethyl acetate, which was concentrated on the rotary evaporator if necessary.

RESULTS

Chemical and functional tests for a corynebacterial siderophore. Bacterial siderophores are compounds that chelate iron with high affinity and promote the uptake of iron by bacteria (20). Under appropriate conditions, the addition of siderophore to the growth medium can stimulate growth and uptake of iron by siderophore-deficient bacterial mutants (14). We have obtained evidence that *C. diphtheriae* produces a compound during growth under low-iron conditions that fulfills all of these requirements for designation as a corynebacterial siderophore.

TABLE 1. Iron-solubilizing activity of culture supernatants from wild-type and mutant strains of *C. diphtheriae*[a]

Bacterial strain	Added iron (μg of Fe^{3+}/ml)	Growth (A_{590})[b]	Iron-solubilizing activity (μg of Fe^{3+} solubilized per ml of supernatant)
C7	0.075	4.0 ± 0.2	66.4 ± 13.9
	1.0	4.9 ± 0.1	32.7 ± 4.9
C7(β) (stock culture)	0.075	3.2 ± 0.7	26.7 ± 11.0
	1.0	3.9 ± 0.5	11.8 ± 0.7
C7(β) (new lysogen)	0.075	4.0 ± 0.8	71.6 ± 11.5
	1.0	5.2 ± 0.5	32.5 ± 11.6
HC6	0.075	0.4 ± 0.1	1.5 ± 1.1
	1.0	0.9 ± 0.5	1.9 ± 1.4

[a] The data are means ±standard deviations for results from three or four different experiments.
[b] Stationary-phase cultures (20 h). A_{590}, Absorbance at 590 nm.

Supernatants from stationary-phase cultures of *C. diphtheriae* C7 and C7(β) grown under low-iron conditions contained significant amounts of iron-solubilizing activity (Table 1). Production of iron-solubilizing activity decreased by at least 50% when the initial concentration of Fe^{3+} added to cultures in deferrated growth medium was increased from 0.075 to 1 μg/ml. We reported previously that iron-solubilizing activity was produced by *C. diphtheriae* during the late-exponential and early-stationary phases of growth in low-iron medium, in a manner analogous to production of diphtheria toxin (13). The time after inoculation at which iron-solubilizing activity appeared in the supernatant decreased in cultures grown at pH 8 and increased in cultures grown at pH 5 (results not shown). These observations indicated that *C. diphtheriae* produced larger amounts of one or more iron-solubilizing compounds in response to iron deprivation. The supernatants from low-iron cultures of *C. diphtheriae* did not give positive Arnow tests for *o*-dihydric phenols (1) or positive Csaky tests for hydroxamates (7). Our stock culture of *C. diphtheriae* C7(β) produced less than half as much iron-solubilizing activity as strain C7, but a new C7(β) strain prepared by lysogenization of C7 with phage β produced as much iron-solubilizing activity as the ancestral C7 strain (Table 1). Other experiments demonstrated that stock cultures of strains C7($\beta^{tox\text{-}201}$), C7($\beta^{tox\text{-}202}$), C7($\beta^{tox\text{-}203}$), C7(β^{tox+}_{cr1}), and C7(γ) produced iron-solubilizing activity in amounts comparable to wild-type C7. These findings indicated that one or more mutation affecting production of iron-solubilizing activity had occurred in C7(β) at some time during the three decades that it has been maintained in the laboratory.

C. diphtheriae HC6 was isolated as a mutant of C7(β) that grew very poorly on deferrated agar medium and formed small colonies on iron-supplemented agar medium. Growth of strain HC6 in liquid medium was poor with or without added iron, and the supernatants of cultures of strain HC6 contained little or no iron-solubilizing activity (Table 1). The properties of strain HC6 were consistent with those of a

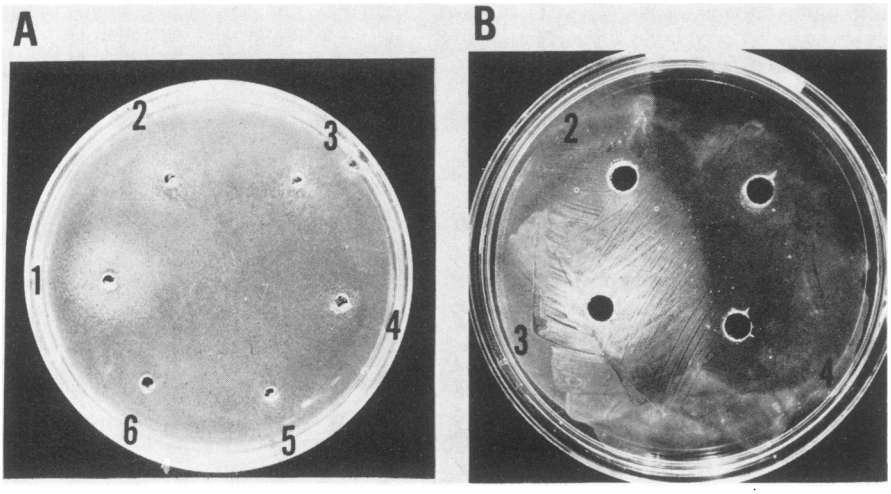

FIG. 1. Bioassay for corynebacterial siderophore. (A) Stimulation of growth of *C. diphtheriae* HC6 on DA-1 agar plates. A 10-μl sample of the undiluted low-iron C7(β) culture supernatant was added to well 1, and 10-μl samples of twofold serial dilutions of the same low-iron conditioned medium were added to wells 2 to 6. (B) Stimulation of growth of strain HC6 on DA-1 plus EDDA agar plates. Samples (20 and 50 μl) of concentrated siderophore from low-iron C7 culture supernatants were added to wells 1 and 2, respectively. Well 3 contained 50 μl of concentrated siderophore plus 200 ng of Fe^{3+}. Well 4 contained 200 ng of Fe^{3+} without siderophore.

siderophore-deficient bacterial mutant. Using HC6 as a bacterial indicator strain, we developed a bioassay to detect the corynebacterial siderophore in supernatants from cultures of *C. diphtheriae* C7 or C7(β) (Fig. 1A). When 10-μl samples of diluted supernatants from low-iron cultures of strain C7(β) were placed in wells in DA-1 agar plates inoculated with strain HC6, growth-stimulating activity was usually detectable to a dilution of 1:16 or 1:32. In contrast, the activity of supernatants from high-iron cultures disappeared at a dilution of 1:2 or 1:4. No growth stimulation was detected when samples of Fe^{3+} solutions or uninoculated medium were added to similar wells in control plates. When the high-affinity iron-chelating agent EDDA was added to the DA-1 agar medium in the bioassay plates, the growth-promoting activity of low-iron conditioned medium decreased dramatically. It was necessary to add both Fe^{3+} and siderophore to the test wells to obtain maximal growth stimulation in DA-1 medium containing EDDA (Fig. 1B). Under similar conditions, the growth of mutant HC6 was not stimulated by iron plus enterochelin, DHBA, or a variety of hydroxamate compounds, including rhodotorulic acid, ferrichrome A, desferrioxamine B, and vibriobactin. In contrast, growth of the HC6 indicator strain was stimulated by iron plus aerobactin. No stimulation of growth by aerobactin was seen without added iron. Low-iron supernatants from cultures of *C. diphtheriae* C7(β) did not stimulate the growth of *A. flavescens* JG9, *S. typhimurium enb*-7, or *S. flexneri* M4243 in agar diffusion bioassays. In control experiments growth of the *S. flexneri* indicator strain was stimulated by purified aerobactin. These observations provided strong evidence for a corynebacterial siderophore that stimulated the growth of *C. diphtheriae* HC6 in the presence of iron and was different from the well-characterized phenolate and hydroxamate siderophores tested.

Next, we demonstrated a functional role for corynebacterial siderophore in the uptake of Fe^{3+} by washed cells of *C. diphtheriae* (Fig. 2). At pH 8.0 the rate of uptake of Fe^{3+} by washed C7(β) cells suspended in low-iron conditioned medium was approximately twice that of cells in a mid-log-phase control culture of the same cell density. In contrast, the rate of iron uptake by washed cells at pH 8.0 in fresh deferrated medium or in high-iron conditioned medium was only half that of the control cells. The observed rates of iron uptake were not changed in the presence of the iron-chelating compound NTA. These results demonstrated that the uptake of Fe^{3+} was mediated by a high-affinity system and that a factor present in low-iron conditioned medium was required for a maximal uptake rate. Unlike wild-type C7(β), mutant HC6 failed to grow in deferrated liquid medium without added iron. To compare iron uptake by C7(β) and HC6 cells, therefore, cultures were grown in low-iron medium supplemented with 0.075 μg of Fe^{3+} per ml. In low-iron conditioned medium at pH 8.0, the rate of iron uptake by washed C7(β) cells grown in medium with 0.075 μg of added Fe^{3+} per ml (Fig. 3) was only one-third that of washed C7(β) cells grown in medium with no added iron (Fig. 2). When $^{59}Fe^{3+}$ was added to growing cultures of strain HC6 at pH 8.0 (Fig. 3) or pH 6.8 (data not shown), the rate of iron uptake was very low. The rate of iron uptake by strain HC6 was similar to that of several previously reported mutants of *C. diphtheriae* C7(β) that were severely defective in iron transport (6). When HC6 cells were suspended in low-iron conditioned medium from strain C7(β), their rate of iron uptake was stimulated to a level at least half that of C7(β) cells under the same conditions (Fig. 3). This stimulation of iron uptake by HC6 cells in low-iron conditioned medium was not prevented by NTA. Enterochelin, DHBA, rhodotorulic acid, ferrichrome A, and desferrioxamine B did not stimulate iron uptake by strain HC6 under these conditions. We did not have sufficient amounts of vibriobactin and aerobactin to test their activity in this system. Iron transport by the mutants of *C. diphtheriae* designated HCl, HC3, HC4, and HC5 described previously (6) was not stimulated by the corynebacterial siderophore present in low-iron conditioned medium (results not shown). These observations demonstrated that corynebacterial siderophore stimulated iron uptake by *C. diphtheriae* mutant HC6 and that mutants of *C. diphtheriae* defective in iron transport could be separated into at least two phenotypically different groups based on their responses to added siderophore.

Partial purification of the corynebacterial siderophore. We purified the siderophore from supernatants of low-iron cul-

tures of the nontoxinogenic C7 strain of *C. diphtheriae*. Siderophore activity was extracted with methanol, followed by precipitation with ethyl acetate, extraction with water, lyophylization, and solution of the residue in water. This concentrated extract had a high level of activity in the plate bioassay with strain HC6, stimulated uptake of Fe^{3+} by strains C7(β) and HC6 at pH 8.0, and contained essentially all of the iron-solubilizing activity of the original culture supernatant (Table 2). Samples of this concentrated extract were used to test a variety of further purification methods, including preparative TLC, column chromatography, and HPLC. The data in Fig. 4 illustrate the results of chromatography on a representative silica column in an ethanol–1 mM ammonium acetate solvent system (80:20) at pH 7.2. Two major peaks were detected by measurement of absorbance at 360 nm. Siderophore activity was detected in fractions 8 to 11, corresponding to peak A. Samples of these fractions were spotted onto TLC plates and shown to give positive reactions with phenolate spray and to fluoresce blue under long wavelength UV light. In contrast, fractions 14 to 17, corresponding to peak B, had no siderophore activity and showed red fluorescence under similar conditions. Pooled fractions from peak B were shown to contain large amounts of coproporphyrin III, whereas fractions from peak A contained very little coproporphyrin III.

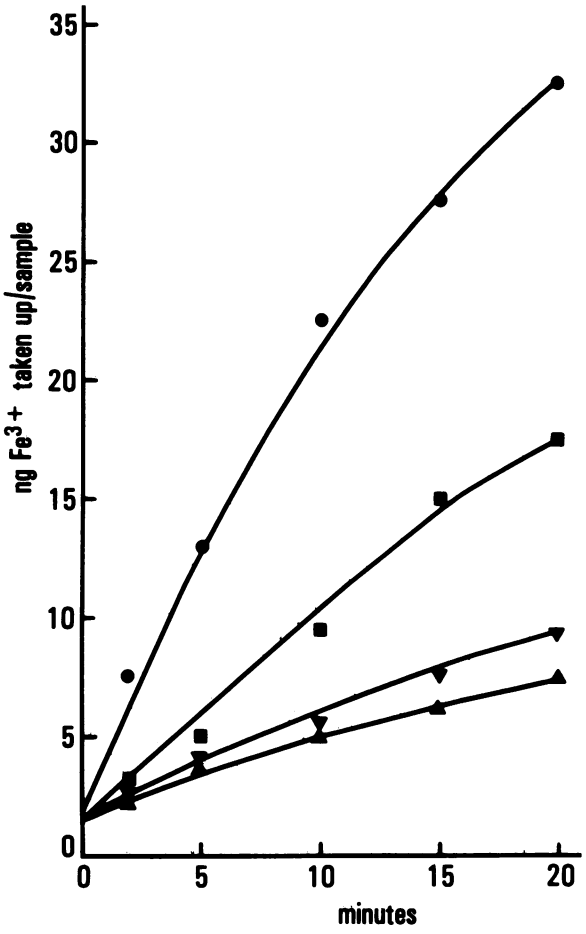

FIG. 2. Requirement for low-iron conditioned medium for maximal iron uptake by *C. diphtheriae* C7(β). Cells of strain C7(β) were grown in deferrated PGT-maltose medium without added iron, and $^{59}Fe^{3+}$ uptake was measured at pH 8.0. Symbols: ■, control culture; ▲, washed cells resuspended in fresh deferrated PGT-maltose medium; ▼, washed cells resuspended in high-iron conditioned medium; ●, washed cells resuspended in low-iron conditioned medium. The addition of 100 mM NTA to control cultures or suspensions of washed cells did not change the rate of uptake of radiolabeled iron.

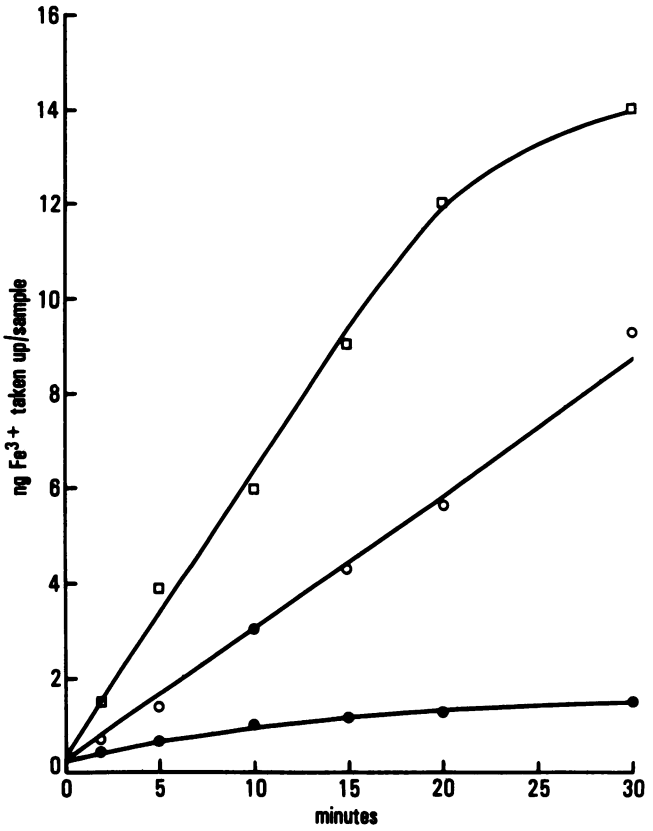

FIG. 3. Stimulation by low-iron conditioned medium of iron uptake by *C. diphtheriae* strain HC6. Cells were grown in deferrated PGT-maltose medium with 0.075 μg of added Fe^{3+} per ml, and uptake of $^{59}Fe^{3+}$ was measured at pH 8.0. Symbols: ●, control cultures of HC6; ○, washed HC6 cells resuspended in low-iron conditioned medium from C7(β) cultures; □, washed C7(β) cells resuspended in low-iron conditioned medium from C7(β) cultures.

A sample of the peak A material from the silica column was analyzed by HPLC on a reverse-phase C18 silica column eluted with a 0 to 50% linear gradient of acetonitrile in water (Fig. 5). Two peaks were detected by analyzing absorbance at 360 nm. The first peak was complex, and pooled material from this peak had both siderophore activity in plate bioassays with HC6 and iron-solubilizing activity. Not enough material was available to test for stimulation of iron uptake in cultures of strain HC6. The second, more hydrophobic peak contained coproporphyrin III but no detectable corynebacterial siderophore. Analysis of pooled fractions from the first peak by TLC showed two UV-absorbing spots with slightly different mobilities (R_f 0.79 and 0.76). The material in the spot with the slower mobility reacted with phenolate spray, although Arnow tests for catechols and Csaky tests for hydroxamates were negative.

DISCUSSION

The possibility that *C. diphtheriae* produces siderophore(s) under low-iron conditions has been considered for

TABLE 2. Purification of corynebacterial siderophore from low-iron conditioned medium of *C. diphtheriae* C7

Sample	Iron-solubilizing activity (μg of Fe^{3+} solubilized/ml)[a]	Stimulation of growth of HC6 (diameter in cm)[b]	Stimulation of iron uptake by strain HC6[c]
Low-iron conditioned medium	78	2.76	+
Concentrated siderophore	1,680 (100%)	3.60	+
Peak A from silica column	742 (88%)	2.94	NT
Peak A from HPLC	300 (81%)	2.31	NT
Peak B from HPLC	36 (19%)	—	NT

[a] Figures in parentheses are estimates of the recovery of iron-solubilizing activity for each step of the purification. Volumes at each step are given in the text. The cumulative recovery of iron-solubilizing activity is the product of the recoveries for each step.

[b] Samples (50 μl) plus 100 ng of Fe^{3+} were added to wells in DA-1 plus EDDA plates, and the growth around wells was measured after 48 h. —, No detectable growth stimulation.

[c] Washed cells were suspended at pH 8.0 in low-iron conditioned medium or in fresh deferrated PGT-maltose medium supplemented with concentrated siderophore. +, Detectable stimulation of iron transport; NT, not tested.

some time (2), but this paper describes the first characterization of a corynebacterial siderophore. We obtained the corynebacterial siderophore in a partially purified form, but the chemical structure of the siderophore has not been determined. The HPLC data indicated that at least two closely related chemical compounds were present in the fractions with siderophore activity. A similar situation was reported for the analysis of pyoverdin activity in *Pseudomonas aeruginosa* (C. D. Cox, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, D11, p. 60). The negative results in Arnow and Csaky tests with the corynebacterial siderophore raised the possibility that it may not be closely related to other well-characterized phenolate and hydroxamate siderophores. This hypothesis was supported by the observation that the corynebacterial siderophore failed to stimulate the growth of the *S. typhimurium enb*-7, *A. flavescens* JG9, and *S. flexneri* M4243 indicator strains. The stimulation of growth of mutant

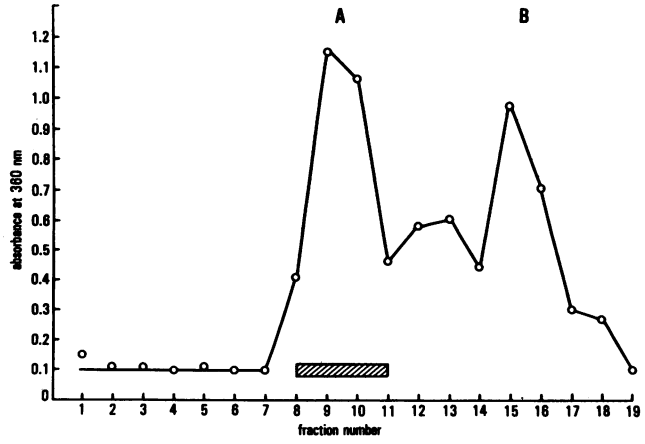

FIG. 4. Chromatography of concentrated siderophore from low-iron conditioned medium from *C. diphtheriae* C7 on a silica gel column. Growth-stimulating activity (▭) was assayed by placing 25-μl samples from each fraction into wells cut in DA-1 plates spread with lawns of strain HC6. Growth stimulation was assessed after 48 h of incubation at 37°C.

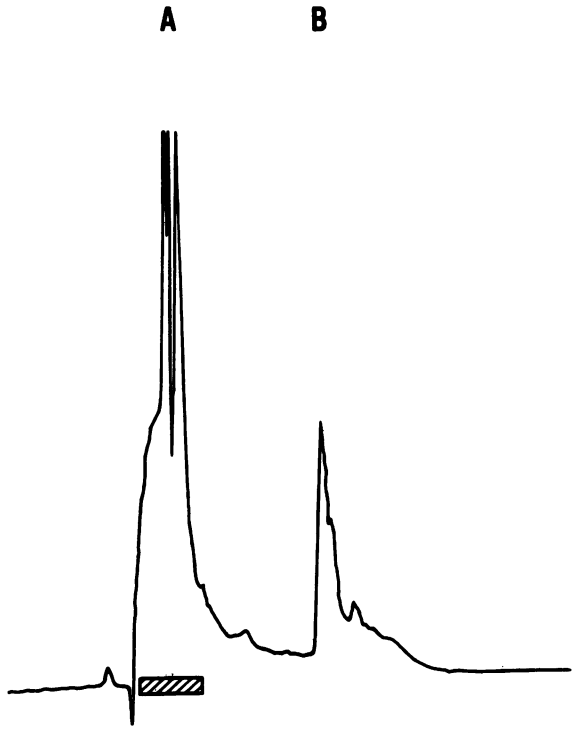

FIG. 5. HPLC of corynebacterial siderophore. A sample of peak A material from Fig. 4 was analyzed as described in the text. The retention time for peak A was 10.8 to 12.5 min; the retention time for peak B was 30.7 min. Growth-stimulating activity (▭) was associated only with the material in peak A and was assayed as described in the legend to Fig. 4.

HC6 by aerobactin indicated that *C. diphtheriae* can utilize exogenous aerobactin as a functional siderophore, but the properties of the corynebacterial siderophore were not identical to those of aerobactin. Our most purified siderophore preparations gave positive reactions with the phenolate spray reagent but not with the Arnow test. The spray reagent detects all phenolate compounds, whereas the Arnow test is selective for aromatic vic-diols in which either the 3- or 4-position is unsubstituted and neither position is sterically blocked (4). Further purification of the corynebacterial siderophore will determine whether it is or is not identical to the phenolate compound in peak A from our HPLC experiments.

A common characteristic of many well-studied bacterial iron transport systems is inhibition of siderophore production under high-iron conditions (19). The titer of corynebacterial siderophore detected in our agar diffusion bioassay with mutant HC6 was 4- to 16-fold greater with low-iron conditioned medium than with high-iron conditioned medium, and stimulation of iron transport by washed cells of *C. diphtheriae* C7(β) or HC6 occurred with low-iron conditioned medium but not with high-iron conditioned medium (Fig. 1 and 2). These observations demonstrated that very little siderophore was produced by *C. diphtheriae* under high-iron conditions. Nevertheless, high-iron conditioned medium had significant amounts of iron-solubilizing activity, equivalent to half that of low-iron conditioned medium (Table 1). It is apparent that compounds other than siderophore contributed significantly to the iron-solubilizing activity of high-iron conditioned medium. In contrast, most of the iron-solubilizing activity of low-iron conditioned medium

copurified with the siderophore (Table 2), and it is likely that siderophore accounted for a large proportion of total iron-solubilizing activity in low-iron conditioned medium. Coproporphyrin III may have some iron-solubilizing activity in our assay system (Table 2 and Fig. 5), but our data provide no evidence that coproporphyrin III can function as a corynebacterial siderophore.

The siderophore-deficient mutant HC6 isolated from C7(β) was severely defective in growth and iron transport. These functional defects were reversed in the presence of conditioned medium or partially purified corynebacterial siderophore. We have isolated revertants of HC6 that behave like wild-type C7(β) with respect to growth, iron uptake, and production of iron-solubilizing activity. These observations support the hypothesis that all of the phenotypic changes in mutant HC6 were pleiotropic effects of a single mutation affecting siderophore production. Under the conditions of our experiments the major pathway(s) for iron uptake by *C. diphtheriae* appears to require the corynebacterial siderophore.

Deficiency of iron in the growth medium of *C. diphtheriae* stimulates the production not only of corynebacterial siderophore but also of diphtheria toxin and coproporphyrin III (2, 5, 23, 33). In *Bacillus subtilis* the accumulation of phenolate siderophores is accompanied by accumulation of coproporphyrin. Although the biosynthetic pathways for siderophores and for coproporphyrin in *B. subtilis* are both regulated by iron, they are not linked by a common control mechanism (25). We are currently investigating the mechanism(s) by which iron regulates the biosynthesis of toxin, coproporphyrin, and siderophore in *C. diphtheriae*.

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