Effects of Iron Limitation on Adherence and Cell Surface Carbohydrates of *Corynebacterium diphtheriae* Strains

Lílian de Oliveira Moreira,1,2 Arnaldo Feitosa Braga Andrade,1 Márcio Damasceno Vale,1 Sônia Maria Silva Souza,1,3 Raphael Hirata, Jr.,1 Lídia Maria Oliveira Buarque Asad,3 Nasser Ribeiro Asad,3 Luiz Henrique Monteiro-Leal,3 José Osvaldo Previato,2 and Ana Luíza Mattos-Guaraldi1*

*Faculdade de Ciências Médicas*1 and *Instituto de Biologia Prof. Roberto Alcântara Gomes,*2 *Universidade do Estado do Rio de Janeiro,* and *Instituto de Biofísica Carlos Chagas Filho,*3 *Universidade Federal do Rio de Janeiro,*2 Rio de Janeiro, Brazil

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Iron limitation may cause bacterial pathogens to grow more slowly; however, it may also stimulate these microorganisms to produce greater tissue damage, given that many virulence factors are controlled by the iron supply in the environment. The present study investigated the influence of low iron availability on the expression of proteins and surface sugar residues of two toxigenic strains of *Corynebacterium diphtheriae* subsp. mitis and evaluated their adherence to human group B erythrocytes and HEp-2 cells. A comparison was made between bacteria grown in (i) Trypticase soy broth (TSB), (ii) TSB treated with dipyridyl to deplete free iron, and (iii) TSB enriched with FeCl3. The effects of iron concentration on adhesive properties were different for strains 241 and CDC-E8392, of the sucrose-fermenting and non-sucrose-fermenting biotypes, respectively. Iron-limited conditions enhanced interaction of strain 241 with erythrocytes and HEp-2 cells. Inhibition assays suggested the involvement of nonfimbrial protein combinations 67-72p on hemagglutination of diphtheria bacilli grown under iron-limited conditions. Conversely, iron limitation inhibited adherence to glass and expression of electron-dense material on the bacterial surface. Lectin binding assays demonstrated a reduction in the number of sialic acid residues and an increase in α-mannose and α-galactose residues on the surfaces of both strains. Thus, iron exerts a regulatory role on adhesive properties of diphtheria bacilli, and low iron availability modulates the expression of *C. diphtheriae* surface carbohydrate moieties. The significant changes in the degree of lectin binding specific for α-mannose, α-galactose and sialic acid residues may have an effect on binding of host cells. The expression of dissimilar microbial virulence determinants may be coordinately controlled by common regulatory systems. For *C. diphtheriae*, the present results implicate regulation of adherence and slime production as part of a global response to iron-limited environmental conditions that includes derepression of genes for the synthesis of cytotoxin and siderophores and for transport of the Fe(III)-siderophore complexes.

The examination of environmental signals controlling virulence is an essential step in comprehension of the underlying strategies that microbes have adopted to become successful pathogenic organisms (15). Iron is essential for bacterial growth, but its availability in the human body is very limited because it is almost entirely complexed with metalloproteins or glycoproteins (3, 24). During infection, the restriction of iron is stronger because the host response to the invading bacteria includes hypoferremia (4). *Corynebacterium diphtheriae* is able to overcome host conditions, in part by producing siderophores or other iron uptake mechanisms that allow it to express virulence factors such as toxins and enzymes. Cytotoxins, siderophores, heme oxygenase (HmuO), and cell surface lipopolysaccharides are regulated by both iron and DtxR protein (18, 20–22).

Epidemic or invasive clones, as well as the atypical sucrose-fermenting biotype, of diphtheria bacilli seem to possess some selective advantage, such as increased virulence or an enhanced ability to colonize and spread (14). Attachment of bacteria is a critical step in the pathogenesis of many infections, particularly in cases where the pathogen is confined to mucosal surfaces. Investigations carried out with Brazilian diphtheria bacillus isolates demonstrated that non-sucrose-fermenting strains preferentially colonize skin lesions and show higher cell surface hydrophobicity than throat-colonizing sucrose-fermenting strains (12, 13). *C. diphtheriae* strains adhere to human erythrocytes and solid surfaces at various intensities. The hemagglutinating activity of nonfimbrial adhesion protein combination 67-72p is influenced by the concentrations of sugar residues expressed on bacterial surface (5). Sialic acid terminal moieties are expressed mainly on the surface of the nonhemagglutinating and highly glass-adherent sucrose-fermenting strain 241 (11). *C. diphtheriae*, which is generally considered to be an extracellular colonizer, also exhibits the ability to survive within cultured epithelial cells (9). The molecular differences observed in bacterial adherence to host cells might correlate with maintenance and dissemination of specific *C. diphtheriae* clones.

The expression of adherence factors by several species is influenced by the iron supply in the environment. Iron restriction in the growth medium promotes slime production by
Staphylococcus aureus (1, 17) and Staphylococcus epidermidis (7), production of mucin-binding adhesins by Pseudomonas aeruginosa (19), and hydrophobicity of and adherence to HEp-2 cells by Vibrio parahaemolyticus (6).

Although studies have reported regulatory roles of iron in the pathogenesis of C. diphtheriae infection, little is known about the actual connection between iron availability and the adhesive properties of diphtheria bacilli. The present study investigated the influence of low iron availability on the expression of proteins and surface sugar residues of two toxigenic strains of C. diphtheriae and evaluated their adherence to human erythrocytes and HEp-2 cells.

**Materials and Methods**

**Bacterial strains and culture conditions.** The toxigenic, sucrose-fermenting C. diphtheriae subsp. mitis strain 241 (from Diphtheria Laboratory of Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil) and the non-sucrose-fermenting strain CDC-E8392 (from the Centers for Disease Control and Prevention, Atlanta, Ga.) were used in this study (14). Microorganisms were stored in GC medium base (Difco Laboratories, Detroit, Mich.) with 20% glycerol at −20°C.

Microorganisms were grown in three different media at 37°C for 24 h without shaking: Trypticase soy broth (TSB) (Difco) (iron content [measured by atomic absorption spectrophotometry] of 0.74 μg ml⁻¹), TSB deprived of iron by addition of the chelating agent 2,2’-dipyridyl (Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 0.5 mM (TSB – Fe) (19) and TSB-enriched with 4.0 mM in 1.0 M FeCl₃ (TSB + FeCl₃) (21). Experiments were also done with microorganisms initially cultured in iron-limited TSB and subcultured in TSB (TSB – Fe) TSB. Bacterial cells were harvested by centrifugation at 10,000 g for 10 min at 4°C. Supernatant and HEp-2 monolayer lysates for each incubation period were determined and expressed as the mean ± standard deviation from three independent experiments performed in triplicate. The adherence index for each incubation period represented the percentage of adherence, calculated as lysate CFU (CFU ml⁻¹) / (lysate CFU ml⁻¹ + supernatant CFU ml⁻¹) × 100.

**Hemagglutination and hemagglutination inhibition assays.** Hemagglutination and hemagglutination inhibition assays were performed with a 0.5% suspension of human group B erythrocytes as previously described (5, 12). The nonfimbrial adhesion protein concentration 67-72p were obtained from C. diphtheriae strain CDC-E8392 grown in TSB by mechanical blending and precipitation by ammonium sulfate (25% and 45% saturation) (5). Hemagglutination inhibition assays were performed in the presence of dilutions of a solution of these protein adhesins (300 μg/ml) at 37°C for 1 h.

**Bacterial autoaggregation assays.** Briefly, microorganisms that remained clumped in TSB medium were considered to be spontaneously autoaggregating bacteria. Aliquots (50 μl) of bacterial suspensions prepared in PBS (pH 6.8) were dropped on a glass slide and observed for aggregation. Nonaggregating bacteria produced turbid suspensions. Spontaneously autoaggregating strains were considered to be highly hydrophobic, and aggregating strains were considered to be moderately hydrophobic (13).

**Assays for adherence to glass.** Briefly, microorganisms were inoculated in glass tubes (13 by 100 mm) containing 4 ml of TSB and incubated for 48 h at 37°C without shaking. The tubes were gently shaken for 5 s, and the supernatants containing bacterial cells that remained nonadherent to the surfaces of the glass tubes were recorded. TSB (4 ml) was then added, and the tubes were reincubated for 48 h. This procedure was repeated twice. The glass-adherent bacteria created a confluent coat of cells on sides of the tube (12).

**SDS-PAGE analysis of crude cells lysates.** Bacterial cells were lysed by treatment with 5 mg of lysosome (grade I; Sigma) ml⁻¹ in PBS for 2 h at 37°C (16). Protein samples were solubilized in cracking buffer (0.5 M Tris-HCl [pH 6.8], 4% sodium dodecyl sulfate [SDS], 20% glycerol, and 0.001% bromophenol blue) by heating at 105°C for 15 min. After cell debris and unbroken cells were cleared by centrifugation at 14,000 × g for 5 min, supernatants were collected and used as crude cell lysates. The protein profiles were observed by SDS–10% polyacrylamide gel electrophoresis (SDS–10% PAGE) (Bio-Rad, Richmond, Calif.) with the Laemmli buffer system (10), and the gels were stained with Coomassie brilliant blue R-250 (Bio-Rad).

**Western blot analysis of bacterial surface proteins.** Bacterial cell surface proteins were labeled with biotin (Sigma) at 37°C for 1 h by previously described methods (8) and washed with PBS (pH 9.0) before preparation of crude cell lysates as described above. Proteins bands obtained in SDS-PAGE were transferred to a nitrocellulose membrane at 100 V and 400 mA for 90 min with a Mini Trans-Blot cell (Bio-Rad). Protein blots were blocked in PBS containing 0.5% Tween 20 with 5% skim milk for 2 h at room temperature, washed three times with PBS-D (pH 7.3), and incubated with streptavidin-peroxidase conjugate (Sigma) diluted 1:5,000 in PBS–0.5% Tween 20 for 30 min at room temperature. The nitrocellulose membrane was washed three times and then reacted with 0.3% hydrogen peroxide, 1 mg of 3,3'-diaminobenzidine ml⁻¹, and 1 mg of imidazole ml⁻¹ for color development (25).

### TABLE 1. Number of viable bacterial cells in supernatant and HEp-2 cell monolayer lysates and adherence index for C. diphtheriae strains grown under standard and iron-limited conditions

<table>
<thead>
<tr>
<th>Strain and incubations time (min)</th>
<th>No. of viable bacterial cells (10⁶)a</th>
<th>Adherence index (%b)</th>
<th>P valuec</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TSB Total</td>
<td>Cell lysate</td>
<td>TSB – Fe Total</td>
</tr>
<tr>
<td>Sucrose-fermenting 241</td>
<td>30</td>
<td>0.84 ± 0.12</td>
<td>0.50 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>1.06 ± 0.28</td>
<td>0.36 ± 0.13</td>
</tr>
<tr>
<td>Non-sucrose-fermenting CDC-E8392</td>
<td>30</td>
<td>0.28 ± 0.08</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.17 ± 0.11</td>
<td>0.12 ± 0.11</td>
</tr>
</tbody>
</table>

a TSB, bacterial growth in standard TSB; TSB – Fe, TSB medium with 0.5 mM dipyridyl; total, number of viable bacterial cells in supernatant plus number of viable bacterial cells derived from lysate of infected HEp-2 monolayers during specific period of interaction; cell lysate, number of viable bacteria obtained from lysate of infected HEp-2 monolayers during periods of specific interaction. Values are medians ± standard deviations from three experiments, with differences lower than 10%.

b Number of viable bacterial cells in lysate of infected monolayers during specific period of interaction × (number of viable bacterial cells in supernatant plus number of viable bacterial cells in lysate of monolayers during specific period of interaction)⁻¹ × 100.

Statistically significant at a P value of <0.01.
Lectin binding studies. Briefly, a bacterial suspension (15 μl) containing 3 \times 10^7 bacteria ml\(^{-1}\) was placed on glass slides, air dried, and fixed in methanol at 22°C for 10 min. The slides were washed in PBS–5% bovine serum albumin (BSA) for 5 min and then incubated with 15 μl of increasing dilutions of fluorescein isothiocyanate-labeled lectins (FITC-lectin) (Sigma) in PBS–5% BSA at 22°C for 30 min. The slides were then washed three times in PBS–5% BSA for 5 min each time, mounted in PBS-glycerol (50%), and observed under a fluorescence microscope (Universal Photomicroscope; Zeiss, Oberkochen, Germany). FITC-lectin binding to bacterial cells was inhibited by preincubation with the specific sugar hapten (0.1 M) (11).

Lectin radioiodination and binding studies. The lectins from *Sambucus nigra* (SNA) and *Canavalia ensiformis* (ConA) were conjugated with 125I-labeled acylating agent in the presence of 0.1 M specific inhibitory sugars (2). Specific activities ranged from 1 \times 10^4 to 3 \times 10^5 cpm of lectin μg\(^{-1}\). For the lectin binding studies, 10^6 bacterial cells were incubated with a range of concentrations of iodinated lectin in PBS–0.5% BSA. The amount of iodinated lectin bound to the cells was determined with a gamma counter (Beckman Instruments, Inc., Palo Alto, Calif.). The specificity of binding was ascertained by performing parallel binding determinations in the presence of 0.1 M concentration of the specific sugar inhibitor for each concentration of lectin used (11).

Transmission electron microscopy (TEM). Bacterial cells (strain 241) were fixed with 0.2% glutaraldehyde at 4°C for 15 min, placed on Formvar-coated grids (200 mesh; Sigma), negatively stained with 1% potassium phosphotungstate, and viewed in an EM 906 Zeiss transmission electron microscope (5).

### RESULTS

#### HEP-2 cell adherence.
Higher levels of adherence to HEP-2 cells by strains 241 (62%) and CDC-E8392 (59%) cultured in standard medium (TSB) were observed within 30 and 120 min of incubation, respectively (Table 1). Iron-limited (TSB – Fe) cultures of strain 241 showed significantly higher levels of adherence to HEP-2 cells than standard (TSB) cultures within 30 and 120 min of incubation (P = 0.001). The CDC-E8392 strain showed similar adherence levels when cultured under standard and iron-limited conditions (P > 0.01). The total number of adherence to glass and autoaggregating activity of *C. diphtheriae* strains grown under different iron conditions\(^*\)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Glass adherence</th>
<th>Autoaggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TSB</td>
<td>TSB – Fe</td>
</tr>
<tr>
<td>Sucrose-fermenting 241</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Non-sucrose-fermenting CDC-E8392</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

\(^*\)TSB, bacterial growth in standard TSB medium; TSB – Fe, TSB medium with 0.5 mM dipyridyl; TSB – Fe(TSB – Fe), initial incubation in TSB with 0.5 mM dipyridyl and reincubation in standard TSB; TSB + FeCl\(_3\), TSB with 4.0 mM to 1 M FeCl\(_3\); +, positive result; ++, strong positive result; –, negative result.

#### Statistical analysis. Results were statistically analyzed with Student’s t test.

#### FIG. 1. Protein profiles of strains CDC-E8392 (A) and 241 (B) of *C. diphtheriae* grown under standard and iron-limited conditions. Total protein was analyzed by SDS–10% PAGE. Lane 1, molecular mass markers; lanes 2 and 4, strains CDC-E8392 and 241, respectively, from standard cultures; lanes 3 and 5, strains CDC-E8392 and 241, respectively, from iron-limited cultures. Arrows indicate proteins bands expressed at high levels, and dots indicate proteins bands that were absent or expressed at low levels in iron-limited cultures.
cells of strain 241 remained essentially unchanged during 120 min of incubation with HEp-2 cells, while the number of CDC-E8392 cells dropped sharply to between 7 and 17% of the inoculums. This seemed to be partially due to medium sensitivity of strain CDC-E8392. However, a differential killing effect by the host cells is also a possibility, which remains under investigation.

Hemagglutination. The hemagglutinating activity (titer of 32) of strain CDC-E8392 was independent of iron availability. For strain 241, binding to erythrocytes (titer of 4) occurred only when the bacteria were grown in iron-limited medium (TSB – Fe) (data not shown). The hemagglutinating activity of strain 241 grown under iron-limited conditions was completely inhibited by subsequent growth of the strain in standard medium (TSB – Fe) TSB. For strain CDC-E8392, there were no differences between the hemagglutinating titers of microorganisms grown in TSB – Fe and TSB. The addition of 4.0 to 1 M FeCl3 to TSB did not result in any significant changes in the hemagglutinating properties of strains 241 (negative) and CDC-E8392 (titer of 32) compared to those seen with TSB. The results of inhibition assays showed that the heterologous 67-72p, obtained from strain CDC-E8392 grown in TSB, inhibited hemagglutination of strain 241 at a minimum concentration of 0.5 µg ml⁻¹. 67-72p also inhibited hemagglutination of strain CDC-E8392 grown in both TSB and TSB – Fe. The data suggest involvement of 67-72p adhesins by strain 241 grown under both standard and iron-limited culture conditions, identifying minor differences in cell wall protein patterns due to iron. The presence of biotin-labeled surface proteins of strain 241 revealed six to eight distinct biotin-labeled surface proteins. The number and relative labeling levels were the same in cells of strain 241 grown under standard and iron-limited culture conditions, identifying minor differences in cell wall protein patterns due to iron. The presence of biotin-labeled surface proteins in the 67- to 72-kDa range suggests expression of 67-72p adhesins by strain 241 grown under both standard and iron-limited culture conditions (data not shown).

Expression of iron-related peptides. Figure 1 shows the SDS-PAGE whole-cell protein profiles of strains CDC-E8392 and 241 grown under standard (lanes 2 and 4) and iron-limited (lanes 3 and 5) conditions. The expression of crude proteins with unknown functions was enhanced in both strains CDC-E8392 (54- and 69 kDa peptides) and 241 (54-kDa peptide) cultured in iron-limited medium. Western blotting analysis of nitrocellulose sheets containing biotinylated bacterial surface proteins of strain 241 revealed six to eight distinct biotin-labeled surface proteins. The number and relative labeling levels were the same in cells of strain 241 grown under standard and iron-limited culture conditions, identifying minor differences in cell wall protein patterns due to iron. The presence of biotin-labeled surface proteins in the 67- to 72-kDa range suggests expression of 67-72p adhesins by strain 241 grown under both standard and iron-limited culture conditions (data not shown).

Fluorescent lectin binding assay analysis. The data present in Table 3 demonstrate that (i) the cells bound representatives of all of the classes of lectins tested; (ii) all of the D-galactose (D-Gal), D-mannose (D-Man), and N-acetylmuraminic (sialic) acid lectins that were bound reacted differentially to bacterial cells depending on the iron conditions of growth; (iii) these differential reactions (when present) were similar in both strains; (iv) N-acetylglucosamine (N-GlcNAc) and N-acetylglucosamine (N-GalNAc) showed iron-sensitive reactivity only in some cases; and (v) while the D-Gal, D-Man, and D-GalNAc lectins showed an increase, the sialic acid and D-GlcNAc lectins showed a decrease in binding at low iron con-

TABLE 3. Activities of lectins of various specificities for C. diphtheriae strains grown under standard and iron-limited conditionsa

<table>
<thead>
<tr>
<th>Lectin specificity</th>
<th>Minimum lectin conc (µg ml⁻¹) for detectable fluorescence of strain:</th>
<th>Effect of iron depletion on lectin binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CDC-E8392 (non-sucrose fermenting)</td>
<td>241 (sucrose fermenting)</td>
</tr>
<tr>
<td></td>
<td>TSB</td>
<td>TSB – Fe</td>
</tr>
<tr>
<td>D-Glcnac-binding lectins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triticum vulgare (wheat germ agglutinin)</td>
<td>62.5</td>
<td>250.0</td>
</tr>
<tr>
<td>Bandieraea simplicifolia (BS-H)</td>
<td>31.2</td>
<td>31.2</td>
</tr>
<tr>
<td>Solanum tuberosum (STA)</td>
<td>250.0</td>
<td>250.0</td>
</tr>
<tr>
<td>D-GalNAc-binding lectins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine max (SBA)</td>
<td>31.2</td>
<td>31.2</td>
</tr>
<tr>
<td>Wisteria floribunda (WFH)</td>
<td>250.0</td>
<td>62.5</td>
</tr>
<tr>
<td>Artocarpus integrifolia (IACA)</td>
<td>31.2</td>
<td>31.2</td>
</tr>
<tr>
<td>D-Gal-binding lectins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Euonymus europaeus (EEL)</td>
<td>&gt;500b</td>
<td>125.0</td>
</tr>
<tr>
<td>Arachis hypogaea (PNA)</td>
<td>&gt;500</td>
<td>62.5</td>
</tr>
<tr>
<td>Ricinus communis I (RCA-I)</td>
<td>62.5</td>
<td>15.6</td>
</tr>
<tr>
<td>D-Man-binding lectins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canavalia ensiformis (ConA)</td>
<td>62.5</td>
<td>7.8</td>
</tr>
<tr>
<td>Lens culinaris (LCL)</td>
<td>125.0</td>
<td>31.2</td>
</tr>
<tr>
<td>Pisum sativum (PSA)</td>
<td>125.0</td>
<td>31.2</td>
</tr>
<tr>
<td>Sialic acid-binding lectin, Sambucus nigra (SNA)</td>
<td>62.5</td>
<td>250.0</td>
</tr>
</tbody>
</table>

a Results are average from three experiments. TSB, bacterial growth in standard TSB medium; TSB – Fe, TSB medium with 0.5 mM dipyridyl.

b No fluorescence was seen at the maximum concentration used.

*on October 30, 2017 by guest*
centrations. The most significant feature of the \(1-M\) binding lectins is the striking of ConA for \(C.\) diphtheriae strains grown under iron-limited conditions, in that this lectin interacted strongly with sucrose-fermenting and non-sucrose-fermenting strains at minimum concentrations of 3.9 and 7.8 \(\mu g\) \(\text{ml}^{-1}\), respectively. The sialic acid-binding lectin from \(S.\) nigra (SNA), as with wheat germ agglutinin lectin, reacted specifically with sucrose-fermenting and non-sucrose-fermenting strains grown in standard TSB at minimum concentrations of 15.6 and 62.5 \(\mu g\) \(\text{ml}^{-1}\), respectively. The FITC-SNA binding to bacterial cells was inhibited by \(N\)-acetylneuraminic acid (5 \(mg\) \(\text{ml}^{-1}\)). \(1-Fu-

**125I-lectin binding analysis.** As shown in Fig. 2A, increasing concentrations of \(125I\)-lectin resulted in binding of the labeled lectin in a saturable fashion. The bacterial growth in iron-limited medium resulted in a marked reduction in \(125I\)-SNA binding, confirming that these bacteria were poorly sialylated. However, a significant increase was observed in the \(125I\)-ConA binding assay. The binding was specific, because it could be reversed with a 0.1 M concentration of the corresponding specific saccharide (data not shown). The binding data in Fig. 2A demonstrate that the differential interaction of the \(C.\) diphtheriae sucrose-fermenting strain with the SNA and ConA lectins (Table 3) correlated with the number of exposed \(125I\)-lectin receptor sites on them. As shown in Fig. 2B, the average number of lectin-binding sites \((n)\) and the lectin association constant \((K_a)\) were determined. For binding of SNA to bacterial cells grown under standard iron conditions, \(n\) was approximately \(3.0 \times 10^5\) cell and \(K_a\) was approximately \(3.9 \times 10^6\) \(M^{-1}\); for binding of ConA to bacterial cells grown under iron-limited conditions, \(n\) was approximately \(5.5 \times 10^5\) cell and \(K_a\) was approximately \(1.8 \times 10^6\) \(M^{-1}\).

**TEM analysis.** Figure 3 demonstrates the structural differences in strain 241 cultured in TSB and TSB – Fe, respectively. The experimental conditions used did not allow obser-

![FIG. 2](image-url)  
**FIG. 2.** (A) Binding of iodinated lectins SNA (■, ○) and ConA (▲, △) to \(C.\) diphtheriae strain 241 grown under standard (■, △) and iron-limited (○, ▲) conditions. (B) For each lectin, the binding data for high-affinity receptors (filled symbols from panel A have been plotted by the method of Steck and Wallach) (23) according to the equation

\[
\frac{C}{[\text{lectin}]} \text{ bound } = \frac{1}{K_n \times 1/[\text{lectin}]} \text{ free } + \frac{1/n}{n} 
\]

where \(C\) is the concentration of bacteria, \(n\) is the number of lectin molecules bound per cell, \(K\) is the lectin association constant, and \([\text{lectin}]\) is the concentration of lectin in molar. All points represent means from triplicate experiments; the standard deviations is less than 10%.

![FIG. 3](image-url)  
**FIG. 3.** Electron micrographs showing structural differences on the surface of the sucrose-fermenting \(C.\) diphtheriae strain 241 grown under standard (A) and iron-limited (B) conditions. Magnifications, \(\times 40,000\) and \(\times 70,500\), respectively.
vation of fimbrial structures. There was no electron-dense material on the bacterial surface of strain 241 grown in iron-limited medium (Fig. 3B).

DISCUSSION

Iron has a regulatory role in the adhesion of diphtheria bacilli to cells of the human respiratory tract (HEp-2 cells) and blood (erythrocytes). As demonstrated in this study, iron restriction in the growth medium enhanced the adhesive properties of the sucrose-fermenting strain 241. This phenomenon was not observed with the non-sucrose-fermenting strain CDC-E8392, suggesting that some, but not all, toxigenic diphtheria bacilli may exhibit an increased ability to colonize epithelial surfaces and spread within the low-iron environment of the host.

Analysis of whole-cell protein profiles showed that iron limitation is able to both inhibit and stimulate protein expression, as previously observed with *S. epidermidis* (26) and *S. aureus* (17, 24), respectively. The roles of most iron-regulated proteins, other than toxin, in the pathogenicity of *C. diphtheriae* remain unknown. Previous transmission electron microscopy studies with immunolabeled colloidal gold-protein A revealed a diffuse distribution of 67-72p on the surfaces of both the hemagglutinating CDC-E8392 and nonhemagglutinating 241 *C. diphtheriae* strains grown in iron-containing medium (5). Here, the results of inhibition assays suggest that the 67-72p protein combination also act as hemagglutinins of *C. diphtheriae*, including strain 241. Western blot analysis demonstrated the binding of 67-72p to membranes of HEp-2 cells, as previously observed with erythrocytes by immunoblotting (5).

Prior investigations demonstrated that differences in degrees of hemagglutination and adherence to glass were related to differences in the expression of surface carbohydrates of *C. diphtheriae*. For strains 241 and CDC-E8392, lectin receptors containing terminal β-GlcNAc, β-GalNAc, β-Gal, β-Man, and sialic acid were identified on surfaces of cells grown in iron-containing medium. Sialic acid residues were expressed mainly on the surface of the nonhemagglutinating and highly glass-adherent strain 241. It is known that sugar residues, particularly sialic acid residues, contribute to the expression of hydrophobic characteristics and adherence to glass by *C. diphtheriae* (11). Here we have demonstrated that iron has a regulatory role in the expression of surface carbohydrate moieties of *C. diphtheriae* strains. We have observed an absolute reduction in the sialic acid residues and a significant increase in the amounts of β-GlcNAc, β-GalNAc, β-Gal, and β-Man residues in both strains. Sialic acid terminal constituents of cell moieties seemed to raise difficulties in *C. diphtheriae* adherence to HEp-2 cells, as previously observed with erythrocytes (11).

Hydrophobic interaction is expected to provide the driving force for host-parasite interaction through the displacement of water and formation of adhesive bonds (6). In the present study, for strain CDC-E8392, iron limitation enhanced bacterial hydrophobicity (autoaggregation) but did not influence the adhesion to human cells. For strain 241, low-iron conditions enhanced bacterial autoaggregation and adherence to erythrocytes and HEp-2 cells but inhibited adherence to glass surfaces. In the same way, previous studies demonstrated that the hydrophobicity and HEp-2 cell adherence of the gram-negative species *V. parahaemolyticus* in iron-limited culture were significantly increased. Those authors suggested that the enhancement of cell adherence of *V. parahaemolyticus* was probably due to the formation of lateral flagella, a cytotoxic factor, or other, unknown factors (6). However, for *C. diphtheriae*, iron limitation intensified autoaggregation and adherence to erythrocytes and HEp-2 cells, possibly by reduction of electrostatic repulsion and/or by increased exposure of 67-72p due to the removal of the sialic acid. Similar observations during bacterial treatment with neuraminidase were previously made (5, 11, 13).

Iron limitation inhibited slime production by strain 241, as suggested by TEM and corroborated by glass and lectin binding assays. Among the structures involved in virulence, bacteria have developed the production of slime, a higher-molecular-mass polymer of carbohydrate that encourages biofilm formation. In the gram-positive species *S. epidermidis*, iron limitation promotes slime production (7). *S. aureus* slime-producing strains preferentially accumulate on surfaces and are responsible for chronic colonization, whereas non-slime-producing strains are responsible for acute infection (24). *C. diphtheriae* slime-producing strains preferentially accumulate on glass surfaces and are responsible for acute respiratory infection, whereas non-slime-producing strains show higher cell surface hydrophobicity and are responsible for colonization of skin lesions (12, 13).

Similar to the case for other human pathogens, under iron-restrictive circumstances *C. diphtheriae* develops alternative metabolic strategies to overcome the environmental conditions. Low iron availability modulates the adhesive properties and expression of surface carbohydrate moieties of strains and consequently may influence the course of *C. diphtheriae* infection.

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