Identification of the Latex Test-Reactive Protein of *Clostridium difficile* as Glutamate Dehydrogenase

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Computer analysis showed that the gene encoding the latex test-reactive protein of *Clostridium difficile* exhibited high levels of homology with glutamate dehydrogenases from various sources. Further analysis demonstrated that the recombinant protein possessed glutamate dehydrogenase activity. Our results show that the protein that reacts in commercial latex tests for *C. difficile* is a glutamate dehydrogenase.

*Clostridium difficile*, which causes pseudomembranous colitis in patients undergoing antibiotic therapy, is an important nosocomial pathogen (8). The disease results from the tissue-damaging toxins (toxin A and toxin B) produced by toxigenic strains of the organism. A number of different tests (tissue culturing, enzyme immunoassay, and latex agglutination) have been developed as aids in the diagnosis of the disease. The tissue culture test, which has been in use for over 10 years, and the enzyme immunoassay, which is currently being evaluated, detect the toxins produced by the organism. The latex agglutination tests marketed by Becton Dickinson Microbiology Systems (Culturette CDI) and Meridian Diagnostics, Inc. (Meritec-*C. difficile*), on the other hand, detect a nontoxic protein (Mr, 43,000, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) produced by *C. difficile* and do not distinguish between toxigenic and nontoxigenic strains (9). The gene encoding the protein was originally cloned in our laboratory to demonstrate the absence of toxic activities associated with the latex test-reactive protein and to show that it is distinct from the toxins of *C. difficile* (7). Strains of *C. sporogenes* and *Peptostreptococcus anaerobius* produce a similar protein.

During the course of our studies of the latex test-reactive protein, we noted that the protein possesses several properties that may yield useful information about clostridial proteins in general. First, the latex test-reactive protein elicits high levels of precipitating antibodies in experimental animals, indicating that it may be useful as a carrier molecule for antibody production. Second, the recombinant protein is produced in high amounts (>10 μg/ml) when expressed in *Escherichia coli*, unlike other clostridial proteins that we have examined (10); thus, the results of studies of this protein may indicate why some recombinant clostridial proteins are expressed much better than others. On the basis of these observations, we continued our studies of the protein in an effort to identify its function. Our experimental approach consisted of (i) sequencing the gene encoding the protein, (ii) identifying the function of the protein by comparing the deduced amino acid sequence with the sequences of other proteins, and (iii) confirming the identified activity by enzymatic analysis.

Cloning and sequencing were done with the *DraI* fragment (pCD5A) encoding the latex test-reactive protein (7) by previously described procedures (3, 11). Expression of the recombinant protein was accomplished with an exonuclease-digested portion of the fragment. The Sequence Analysis Software Package developed by the Genetics Computer Group at the University of Wisconsin was used for sequence analysis. The National Biomedical Research Foundation Protein Sequence Data Base was used for analysis of the deduced amino acid sequence.

For N-terminal sequencing, flasks containing 500 ml of Luria-Bertani medium were inoculated with *E. coli* JM109/pCD5A carrying the gene insert and incubated at 37°C with shaking (200 cycles per min on a gyratory shaker) for 48 h. The cells were collected by centrifugation, suspended in 20 ml of 0.05 M Tris-Cl buffer (pH 7.5), and lysed with a French pressure cell. The debris was removed by centrifugation, and the lysate containing the recombinant protein was passed through a 0.45-μm-pore-size membrane and stored at 4°C. The recombinant protein was purified by gel filtration chromatography on Ultrogel AcA22. Fractions containing the recombinant protein were identified by fused rocket immunoelectrophoresis (1) with rabbit antiserum against the latex test-reactive antigen (7), and the N-terminal sequence was determined by previously described methods (3, 12).

Glutamate dehydrogenase activity was measured at 340 nm with reaction mixtures containing 1 ml of 300 mM potassium phosphate buffer (pH 8), 0.5 ml of 300 mM glutamic acid neutralized to pH 7.5, 0.45 ml of 1 mM NAD, 1 ml of deionized water, and 0.05 ml of test sample. Bovine liver 1-glutamate dehydrogenase (Sigma Chemical Co.) served as the positive control. The protein concentration was determined by use of Coomassie protein assay reagent with bovine serum albumin as the standard (Pierce, Rockford, Ill.).

In our previous cloning studies of the latex test-reactive protein (7), a 2.5-kb *DraI* restriction endonuclease fragment containing the gene was inserted into the *HinClII* site in pUC19 and expressed in *E. coli* JM109. When the 2.5-kb fragment was sequenced, an open reading frame of about 1.2 kb was identified at the 3' end of the insert. Immunodiffusion analysis of the protein expressed by the open reading frame confirmed that the protein reacted specifically with antiserum against the latex test-reactive antigen (data not shown). The gene sequence and the deduced amino acid sequence are shown in Fig. 1. The start site was confirmed by N-terminal sequencing of the expressed recombinant protein. The pro-
| ATG | TCA | GGA | AAA | GAT | CTA | AAT | GTC | TCC | GAC | ATG | GCG | CAA | TCT | CAA | GTA | AAA | AAT | GCA | TGT | GAT | AAA | TTA | GCT | ATG | GAA | CCA | GCA | GTT | TAT |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| H   | S   | G   | K   | D   | V   | N   | F   | E   | M   | A   | Q   | S   | Q   | V   | K   | N   | A   | C   | D   | K   | L   | G   | H   | E   | F   | A   | V   | Y   | 90
| GAA | TTA | TTA | AAA | GAA | CTT | ATG | AGA | GTT | ATA | GAG | GGT | TCC | ATT | CCA | GTA | AAA | AAT | GAT | GAT | GAT | GGT | TCT | ATA | AAA | AAT | TTT | AAA | GGA | TTT | AGA | 180
| E   | L   | L   | K   | E   | P   | M   | R   | V   | I   | S   | P   | V   | K   | H   | M   | D   | D   | G   | S   | I   | K   | T   | F   | K   | G   | F   | R   | 270
| TCA | CAA | CAT | AAT | GAT | CAA | GTA | GGG | CCA | ACA | AAA | AGT | GTT | ATA | AGA | AGA | TTG | CAT | CAA | AAT | GTT | TCA | AGA | GAG | GAA | GAA | TTA | AAA | GCT | TTA | TCT | ATA | 270
| S   | Q   | H   | N   | D   | A   | V   | G   | P   | T   | K   | G   | G   | I   | R   | F   | H   | Q   | N   | V   | S   | R   | D   | E   | V   | K   | A   | L   | I   | S   | 360
| TGC | ATG | ACT | TCC | AAG | TGT | TCT | TCA | AGA | GTA | AGA | AGA | AGA | CCA | ACT | TTA | CCT | CCA | CCA | CCA | GGT | TAT | GCA | ACT | AAA | TTA | AAA | TTA | AGA | GGT | AGT | GAG | CCA | GAG | GTA | AGA | TAT | ACT | 450
| W   | M   | T   | F   | K   | C   | S   | V   | T   | G   | ATA | CCA | AGA | AAA | GTT | ATA | CCA | TCA | CCA | CCA | CCA | AGA | AAA | CCA | AAA | TTA | AAA | TTA | AGA | GGT | AGT | GAG | CCA | CCA | GGT | TAT | CCA | ACT | 540
| TTT | GGT | GGT | TCT | TTA | GCA | AGA | AGA | GCT | GCA | ACT | ATG | AAC | TTA | ACT | GGA | CAA | AGT | TCT | ATA | GGT | GGT | ATA | ACT | AGT | AAA | CCA | GTT | GAA | AAA | CAA | TTT | AGT | AAA | CCA | GCT | GCT | GCT | 630
| G   | G   | G   | S   | L   | G   | R   | T   | A   | T   | G   | F   | C   | V   | A   | T   | R   | A   | T   | E   | A   | A   | A   | K   | L   | G   | C   | I   | D   | M   | 720
| AAA | AAA | CCA | AAA | ATA | AGA | GGT | CCA | GAA | AGC | GCT | TAT | ACA | GTC | ATC | GCA | AAA | GGT | GAT | GGT | CTG | GGT | ACT | GTT | GCA | GGT | GCT | GCT | GCT | AAG | AGA | CCA | AGA | GCT | GCT | GCT | GCT | 810
| ATG | GCT | GAA | TGC | TGT | AAA | TCA | GAA | GCT | TCT | TAT | ATA | TAC | AAT | GAA | AAA | GGT | TTA | GAT | CAA | GCT | GAG | ATG | TTA | GAT | ATG | AAA | GAA | AAA | GGT | TTA | GAT | TAT | ATA | TGA | TAT | 1080
| GAT | GAA | GTA | TTT | CCA | GGA | ATA | GGT | CCT | AGC | GAT | GTA | AGA | GAT | TCT | CTA | CCA | CCA | CCA | CCA | CCA | ACT | CTA | CTA | TAT | TTT | TTA | TTA | GGT | GAG | TTT | GAT | GAA | TTA | GAG | GGA | TTT | TTA | 1250
| I   | K   | E   | E   | Y   | N   | V   | T   | M   | R   | E   | A   | A   | Y   | H   | S   | I   | K   | K   | V   | A   | E   | A   | H   | K   | L   | R   | G   | W   | 1260

**FIG. 1.** Nucleotide and deduced amino acid sequences of the gene encoding the latex test-reactive protein of *C. difficile.*
The C. difficile glutamate dehydrogenase is shown in parentheses, 5' over, accompanied by various glutamines with different internal boxes showing regions that are not homologous with the protein from C. difficile. The boxed region of homology of the various glutamine dehydrogenases with different glutamine dehydrogenases is shown in parentheses, 5' over, accompanied by various glutamines with different internal boxes showing regions that are not homologous with the protein from C. difficile.

FIG. 2. Comparison of deduced amino acid sequences of glutamine dehydrogenases from various sources. The amino acids are shown in parentheses, 5' over, accompanied by various glutamines with different internal boxes showing regions that are not homologous with the protein from C. difficile.

FIG. 3. Analysis of the deduced amino acid sequence of the latex test-reactive protein with the National Biomedical Research Foundation Protein Sequence Database. The deduced amino acid sequence was compared with the sequence of C. difficile glutamate dehydrogenase. The protein from C. difficile glutamate dehydrogenase (4, 5, and 10) was analyzed using the Clustal program. The deduced amino acid sequence was compared with the sequence of C. difficile glutamate dehydrogenase (4, 5, and 10) was analyzed using the Clustal program. The deduced amino acid sequence was compared with the sequence of C. difficile glutamate dehydrogenase (4, 5, and 10) was analyzed using the Clustal program. The deduced amino acid sequence was compared with the sequence of C. difficile glutamate dehydrogenase (4, 5, and 10) was analyzed using the Clustal program. The deduced amino acid sequence was compared with the sequence of C. difficile glutamate dehydrogenase (4, 5, and 10) was analyzed using the Clustal program. The deduced amino acid sequence was compared with the sequence of C. difficile glutamate dehydrogenase (4, 5, and 10) was analyzed using the Clustal program.
regulatory mechanisms. These enzymes are highly conserved and exhibit an extremely low rate of point mutations relative to many other proteins (2). The glutamate dehydrogenase of *C. difficile*, which has been of interest because of its usefulness as a diagnostic marker for the presence of the organism in fecal specimens, also is highly conserved. Glutamate dehydrogenases from other clostridia, including *C. butyricum*, *C. kluyveri*, and *C. symbiosum*, have been studied, but their sequences have not been determined (6, 19).

Nucleotide sequence accession number. The GenBank accession number for the sequence reported here is M65250.

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REFERENCES


Letter to the Editor

Confirmation that the Latex-Reactive Protein of Clostridium difficile Is a Glutamate Dehydrogenase

In their recent article (3), Lyerly et al. described the gene and the deduced amino acid sequence of the latex-reactive protein (LRP) of Clostridium difficile. They noted its similarity to glutamate dehydrogenase (GDH) enzymes from several other sources and the apparent enzymatic activity of the gene product in a lysate of the host cell (Escherichia coli). The enzyme appears to be essential, as both toxic and nontoxic isolates in this study expressed GDH activity in purified LRP preparations. Moreover, all isolates and strains of C. difficile examined to date express LRPs. In addition, immunologically cross-reactive antigens in C. sporogenes, Bacteroides asaccharolyticus, Peptostreptococcus anaerobius, and certain proteolytic strains of Clostridium botulinum have been reported (2). We have noted cross-reactions only with C. sporogenes by using antiserum raised to the C. difficile LRP. While the presence of the LRP in all isolates of C. difficile would suggest that it is not related to pathogenicity, a more extensive study on relative GDH activities in various strains and isolates may now be warranted. Accolades go to Lyerly et al. for a fine piece of molecular sleuthing!

REFERENCES

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TABLE 1. GDH activity of LRPs

<table>
<thead>
<tr>
<th>Source of purified LRP or GDH</th>
<th>Conc (µg/3-ml reaction)</th>
<th>Proteins produced</th>
<th>GDH sp acta (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. difficile VPI 10463</td>
<td>9.4</td>
<td>+</td>
<td>1.57</td>
</tr>
<tr>
<td>C. difficile VPI 11186</td>
<td>2.46</td>
<td>+</td>
<td>7.01</td>
</tr>
<tr>
<td>C. sporogenes ATCC 3584</td>
<td>117.5</td>
<td>+</td>
<td>0.996</td>
</tr>
<tr>
<td>Bovine GDH (Boehringer-Mannheim)</td>
<td>20.0</td>
<td>NA</td>
<td>2.97</td>
</tr>
</tbody>
</table>

a Activity is expressed as A410/min/mg of protein. Concentrations of purified LRPs are based on the A260 divided by the extinction coefficient (10.7 for a 1% solution) for Clostridium subterminale GDH (5) and divided by 9.3 for bovine GDH (1). Assay conditions are as described by Lyerly et al. (3).

b -Ab, without antibody; +Ab, with protein A-purified rabbit anti-C. difficile LRP (18.8 µg).

c ND, not done.

d NA, not applicable.

JM109(pCDS5A). In an effort to confirm their observation that the LRP of C. difficile is GDH, we tested several purified preparations of LRPs for GDH activity (Table 1). LRPs from highly toxigenic (10463) and nontoxigenic (11186) strains of C. difficile had GDH specific activities greater than or comparable to that of the control bovine GDH (Boehringer-Mannheim). The GDH activity of the C. difficile 11186 LRP was blocked by a protein A-purified rabbit anti-C. difficile LRP. Interestingly, the LRP purified from Clostridium sporogenes (ATCC 3584) also had GDH activity, albeit with a lower specific activity. Inhibition by the rabbit anti-C. difficile LRP was less than that observed with C. difficile LRPs (9 versus 69%, respectively); however, the molar level of antibody was kept constant while a higher level of C. sporogenes LRP was required to accurately measure activity. The C. sporogenes LRP is only weakly reactive in the latex test for C. difficile. Rabbit anti-LRP had no effect on bovine GDH activity. These data support and extend the conclusion by Lyerly et al. that the LRP of C. difficile is a GDH. While it is possible that the GDH activities represent an impurity in the purified LRP preparations, this seems unlikely, as the observed specific activity of the C. difficile GDH (from strain 11186) exceeded that of the commercial bovine preparation. In addition, the subunit molecular weight determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and on Western immunoblots has consistently been 43,000 in our laboratory as well as that of others (2). Finally, the experimental approach taken in this study (purification and specific immunoinhibition) was quite different from that of Lyerly et al. (3), while the results are in agreement.

The proposed role of GDH in bacterial ammonia utilization (4) in generating amino acids has not been established for C. difficile. The enzyme appears to be essential, as both toxic and nontoxic isolates in this study expressed GDH activity in purified LRP preparations. Moreover, all isolates and strains of C. difficile examined to date express LRPs. In addition, immunologically cross-reactive antigens in C. sporogenes, Bacteroides asaccharolyticus, Peptostreptococcus anaerobius, and certain proteolytic strains of Clostridium botulinum have been reported (2). We have noted cross-reactions only with C. sporogenes by using antiserum raised to the C. difficile LRP. While the presence of the LRP in all isolates of C. difficile would suggest that it is not related to pathogenicity, a more extensive study on relative GDH activities in various strains and isolates may now be warranted. Accolades go to Lyerly et al. for a fine piece of molecular sleuthing!
Author's Reply

The latex-reactive protein of *Clostridium difficile* has proven to be quite an interesting molecule to us primarily because of our studies on the toxins of the organism. At first this protein was confused with the enterotoxin, toxin A, of the organism, and this confusion ultimately led to our efforts to identify its function. The protein has continued to be a useful marker for the presence of *C. difficile* in fecal specimens. It is important, however, that clinical microbiologists be aware that the protein is produced by nontoxigenic strains which may be present in fecal specimens and which have no apparent role in *C. difficile* disease.

It is always nice when different scientific approaches give the same answer—in this instance, the identification of the latex-reactive protein as a glutamate dehydrogenase. At last, we can call the latex-reactive protein by its true name. We thank David Willis and Jeffrey Kraft of Meridian Diagnostics, Inc., for sharing their findings. Hopefully, this information will be useful to scientists interested in pursuing research on the metabolism of this organism.

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