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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 CDT) and Meridian Diagnostics, Inc. (Meritec-*C. difficile*), on the other hand, detect a nontoxic protein (M_r 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 *Dra*I 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-HCl 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 L-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 *Dra*I restriction endonuclease fragment containing the gene was inserted into the *Hinc*II 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-

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1    ATG TCA GGA AAA GAT GTA AAT GTC TTC GAG ATG GCG CAA TCT CAA GTA AAA AAT GCA TGT GAT AAA TTA GGT ATG GAA CCA GCA GTT TAT   90
       M   S   G   K   D   V   V   N   F   E   M   A   Q   S   Q   V   K   N   A   C   D   K   L   G   M   E   P   A   V   Y

91   GAA TTA TTA AAA GAA CCT ATG AGA GTT ATA GAG GTT TCA ATT CCA GTA AAA ATG GAT GAT GGT TCT ATA AAA ACT TTT AAA GGA TTT AGA  180
       E   L   L   K   E   P   M   R   V   I   E   V   S   I   P   V   K   M   D   D   G   S   I   K   T   F   K   G   F   R

181  TCA CAA CAT AAT GAT GCA GTA GGG CCA ACA AAA GGT GGA ATA AGA TTC CAT CAA AAT GTT TCA AGA GAC GAA GTA 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   S   I

271  TGG ATG ACT TTC AAG TGT TCT GTA ACA GGT ATA CCA TAT GGT GGA GGT AAA GGT GGA ATA ATA GTA GAT CCA TCA ACT TTA TCT CAA GGT  360
       W   M   T   F   K   C   S   V   T   G   I   P   Y   G   G   G   K   G   G   I   I   V   D   P   S   T   L   S   Q   G

361  GAA TTA GAA AGA TTA AGT AGA GGA TAC ATA GAT GGA ATA TAT AAA TTA ATA GGT GAG AAA GTT GAC GTT CCT GCA CCA GAC GTA AAT ACT  450
       E   L   E   R   L   S   R   G   Y   I   D   G   I   Y   K   L   I   G   E   K   V   D   V   P   A   P   D   V   N   T

451  AAT GGT CAA ATA ATG TCT TGG ATG GTT GAT GAG TAC AAT AAA TTA ACT GGA CAA AGT TCT ATA GGT GTT ATA ACT GGT AAG CCA GTT GAA  540
       N   G   Q   I   M   S   W   M   V   D   E   Y   N   K   L   T   G   Q   S   S   I   G   V   I   T   G   K   P   V   E

541  TTT GGT GGT TCT TTA GGA AGA ACA GCT GCT ACT GGA TTT GGT GTT GCT GTT ACA GCA AGA GAA GCT GCT GCT AAA TTA GGA ATA GAT ATG  630
       F   G   G   S   L   G   R   T   A   A   T   G   F   G   V   A   V   T   A   R   E   A   A   A   K   L   G   I   D   M

631  AAA AAA GCT AAA ATA GCT GTT CAA GGT ATA GGA AAC GTT GGT TCT TAT ACA GTT CTT AAC TGT GAA AAA CTT GGT GGT ACT GTT GTA GCT  720
       K   K   A   K   I   A   V   Q   G   I   G   N   V   G   S   Y   T   V   L   N   C   E   K   L   G   G   T   V   V   A

721  ATG GCT GAA TGG TGT AAA TCA GAA GGT TCT TAT GCT ATA TAC AAT GAA AAT GGT TTA GAT GGT CAA GCT ATG TTA GAT TAT ATG AAA GAA  810
       M   A   E   W   C   K   S   E   G   S   Y   A   I   Y   N   E   N   G   L   D   G   Q   A   M   L   D   Y   M   K   E

811  CAT GGT AAC TTA TTA AAC TTC CCA GGA GCT AAG AGA ATA TCT TTA GAA GAG TTC TGG GCT TCA GAT GTT GAT ATA GTA ATA CCA GCT GCA  900
       H   G   N   L   L   N   F   P   G   A   K   R   I   S   L   E   E   F   W   A   S   D   V   D   I   V   I   P   A   A

901  TTA GAA AAC TCT ATA ACT AAA GAA GTT GCT GAA TCT ATA AAA GCT AAA TTA GTT TGT GAG GCT GCT AAT GGA CCA ACT ACT CCA GAG GCT  990
       L   E   N   S   I   T   K   E   V   A   E   S   I   K   A   K   L   V   C   E   A   A   N   G   P   T   T   P   E   A

991  GAT GAA GTA TTT GCT GAA AGA GGA ATA GTT CTT ACT CCA GAT ATA TTA ACT AAT GCT GGT GGA GTT ACA GTT TCT TAC TTT GAG TGG GTA 1080
       D   E   V   F   A   E   R   G   I   V   L   T   P   D   I   L   T   N   A   G   G   V   T   V   S   Y   F   E   W   V

1081 CAA AAC TTA TAT GGA TAC TAC TGG TCA GAA GAA GAA GTA GAG CAA AAA GAA GAA ATA GCT ATG GTT AAA GCA TTT GAA TCT ATT TGG AAA 1170
       Q   N   L   Y   G   Y   Y   W   S   E   E   E   V   E   Q   K   E   E   I   A   M   V   K   A   F   E   S   I   W   K

1171 ATC AAA GAA GAG TAC AAT GTT ACA ATG AGA GAA GCT GCT TAC ATG CAT TCA ATT AAG AAA GTT GCT GAA GCT ATG AAA TTA AGA GGA TGG 1260
       I   K   E   E   Y   N   V   T   M   R   E   A   A   Y   M   H   S   I   K   K   V   A   E   A   M   K   L   R   G   W

1261 TAC TAA
       Y   *
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FIG. 1. Nucleotide and deduced amino acid sequences of the gene encoding the latex test-reactive protein of *C. difficile*.

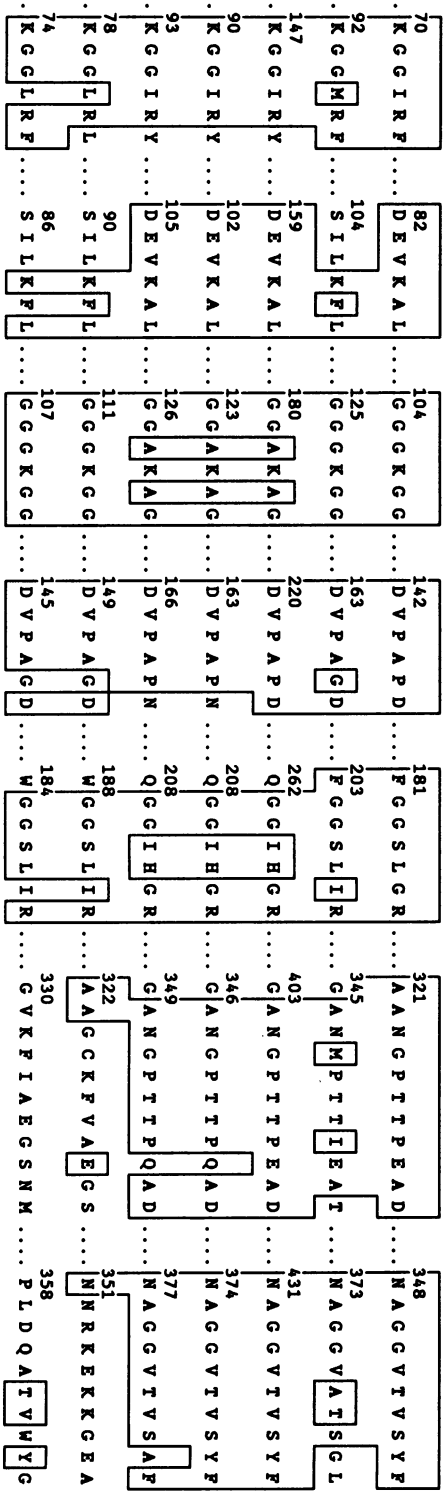

FIG. 2. Comparison of deduced amino acid sequences of glutamate dehydrogenases from various sources. The external boxes show regions of homology with the protein from *C. difficile*. The internal boxes show regions that are not homologous with the protein from *C. difficile*. The actual percentages of homology of the various glutamate dehydrogenases with the *C. difficile* glutamate dehydrogenase are shown in parentheses. *S. cerevi*, *Saccharomyces cerevisiae*.

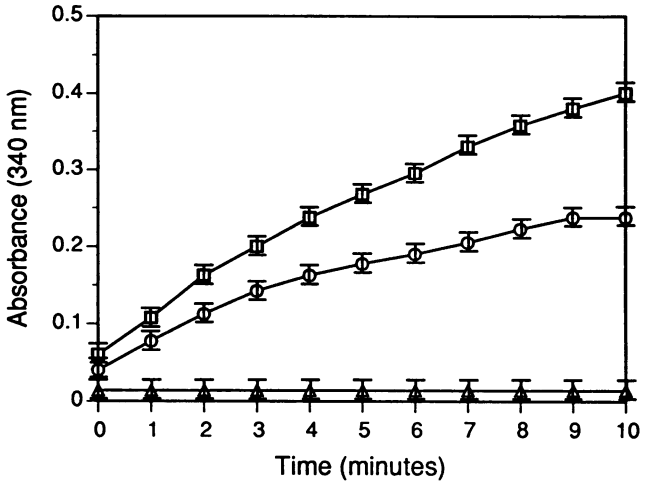

FIG. 3. Analysis of the recombinant protein for glutamate dehydrogenase activity. Lysates were prepared from *E. coli* DH5α containing pUC19 (△) and *E. coli* DH5α containing pUC19 with the gene encoding the latex test-reactive protein of *C. difficile* (○). Assay mixtures contained ca. 0.75 mg of lysate protein. Bovine liver L-glutamate dehydrogenase (17.5 μg containing 0.023 U) was used as the positive control (□). Assays were performed in triplicate. The bars denote standard deviations.

tein consists of 421 amino acids with a deduced molecular weight of 46,015.

Analysis of the deduced amino acid sequence of the latex test-reactive protein with the National Biomedical Research Foundation Protein Sequence Data Base revealed that this protein has high levels of homology with glutamate dehydrogenases (4, 5, 13–18) from various other sources (Fig. 2). On the basis of this finding, we analyzed the recombinant protein for glutamate dehydrogenase activity (Fig. 3). A lysate prepared from cultures of *E. coli* expressing the latex test-reactive protein possessed glutamate dehydrogenase activity, whereas a lysate prepared from cultures not expressing the protein were negative in the assay.

Our results demonstrate that the latex test-reactive protein of *C. difficile* is a glutamate dehydrogenase. Under denaturing conditions, the protein exhibits an M_r of 43,000, as determined by SDS-PAGE, and this estimated molecular weight correlates well with the molecular weight of 46,015 deduced from the gene sequence. Under nondenaturing conditions, however, the protein exhibits an M_r of >200,000, indicating that it exists as an aggregate of subunits. Glutamate dehydrogenases from other sources have also been shown to exist as subunit aggregates, usually as hexamers (4, 5, 13–18). The findings that the native enzyme from *C. difficile* has a high molecular weight and that it is highly immunogenic probably help to explain why this protein was originally confused with toxin A. Toxin A preparations containing small amounts of contaminating glutamate dehydrogenase would elicit high levels of antibody against the protein in addition to antibodies against the toxin. Therefore, latex reagents utilizing these antibodies would not distinguish between toxigenic and nontoxigenic isolates, since nontoxigenic isolates produce as much glutamate dehydrogenase as do toxigenic isolates.

Glutamate dehydrogenases, which reversibly catalyze the amination of α-ketoglutarate to glutamate and the deamination of glutamate to α-ketoglutarate, have been of interest because of their role in nitrogen metabolism and their

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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