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# Survey of Neuraminidase Production by *Clostridium butyricum*, *Clostridium Beijerinckii*, and *Clostridium difficile* Strains from Clinical and Nonclinical Sources

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**Neuraminidase production was investigated in 57 *Clostridium butyricum* strains, 16 *Clostridium beijerinckii* strains, and 25 *Clostridium difficile* strains. Neuraminidase activity was found only in *C. butyricum* strains originating from one human newborn with neonatal necrotizing enterocolitis, two newborns with hemorrhagic colitis, one infected placenta, and one adult with peritonitis. It was concluded that neuraminidase was not a major virulence factor in *C. butyricum* strains.**

Bacterial overgrowth in the intestine of human newborns is an important factor in the pathogenesis of neonatal necrotizing enterocolitis (NNE) (3). Various microorganisms have been associated with NNE including coronavirus, rotavirus, enterovirus, *Escherichia coli*, *Klebsiella* sp., *Enterobacter* sp., *Pseudomonas* sp., *Salmonella* sp., *Clostridium perfringens*, *Clostridium difficile*, and *Clostridium butyricum* (12). *C. butyricum* was involved in several cases of NNE (11, 18, 23, 24). We showed that chickens monoassociated with *C. butyricum* develop cecal lesions similar to those found in NNE (19). *C. difficile* produces a cytotoxin and an enterotoxin, is known to cause pseudomembranous colitis, and was isolated from some NNE outbreaks (5, 10, 23).

Erythrocyte alterations induced by neuraminidase were observed in 9 of 26 newborns with NNE by Seger et al. (22) and in 4 of 20 newborns by Novak (16). Neuraminidase-producing clostridia were isolated in some cases. These authors suggested that neuraminidase-producing bacteria were involved in the pathogenesis of NNE (16, 22), as some *Clostridium* and *Bacteroides* spp. are known to produce extracellular neuraminidase (1, 7, 8). The aim of this work was to investigate the incidence of neuraminidase production in *C. butyricum*, *Clostridium beijerinckii*, and *C. difficile* strains isolated from clinical and nonclinical sources.

The bacterial strains used are listed in Tables 1 and 2. The isolates were identified as *C. butyricum*, *C. beijerinckii*, and *C. difficile* by methods previously described (2, 15). *C. perfringens* type A strain N5 and *Bacteroides fragilis* ATCC 9343 were used as neuraminidase-producing reference strains.

The strains were grown anaerobically at 37°C for 24 h in Trypticase (BBL Microbiology Systems, Cockeysville, Md.)- yeast extract-glucose (TYG) broth (15). Peptone water according to Fraser (7) (peptone [Labosi, Paris, France], 50 g/liter; NaCl, 5 g/liter, pH 7.4) and defined D medium (13) were also used with different incubation times. Bacterial growth was determined by measuring optical density at 600 nm. In some cases, 1% (wt/vol) porcine stomach mucin (Sigma Chemical Co., St. Louis, Mo.) was added. Hemin (5 μg/ml) was added in TYG broth for *B. fragilis* growth.

*C. butyricum* CB1002 isolated from severe NNE (18) was used for preparation of cell extracts. Bacterial cells from 24-h-old TYG cultures with or without mucin were removed by centrifugation and disrupted with 100-μm-diameter glass beads in a Braun apparatus (17).

Neuraminidase activity was measured by incubating, for 30 min at 37°C, 50 μl of culture supernatant concentrated five times by lyophilization with 100 μl of fetuin (10 mg/ml) (Serva, Heidelberg, Federal Republic of Germany) in phosphate-buffered saline (pH 6) containing 9 mM $Ca^{2+}$. The reaction was stopped by the addition of 0.1 ml of 0.2 M sodium periodate in 9 mM *ortho*-phosphoric acid. Enzymatically released *N*-acetylneuraminic acid was determined by the method of Warren (25). One enzymatic unit was the amount of enzyme that released $10^{-9}$ M *N*-acetylneuraminic acid per min at 37°C.

Effects of growth medium and incubation time on neuraminidase activity were determined for two neuraminidase-producing and for one non-neuraminidase-producing *C. butyricum* strains (Fig. 1). Bacterial growth and neuraminidase activity were higher in TYG broth than in D and peptone water media. Neuraminidase activity increased with bacterial growth, and the highest values were observed after 18 h of incubation. In D and peptone media, maximal neuraminidase activity occurred 1 or 2 days after maximal growth. *C. butyricum* CB1002 showed a neuraminidase activity lower than 2.5 enzyme units per ml in TYG broth, so this value was not considered significant.

Of 57 *C. butyricum* strains, 5 (8.7%) exhibited neuraminidase activity, but 16 *C. beijerinckii* strains did not show neuraminidase activity (Table 1). All neuraminidase-producing *C. butyricum* strains were from clinical sources: 1 of 23 (4.3%) strains was isolated from the feces of newborns with NNE, 2 of 16 (12.5%) strains were isolated from the feces of newborns with hemorrhagic colitis, 1 strain was isolated from an infected placenta, and 1 was isolated from an adult with peritonitis. The neuraminidase titer ranged from 7 to 16 enzyme units per ml. *C. perfringens* type A strain N5 and *B. fragilis* ATCC 9343 produced 15 and 17 enzyme units per ml, respectively, under the same experimental conditions. The remaining *C. butyricum* strains were from feces of newborns with acute diarrhea or without disease, from dairy products, and from culture collections, and they did not exhibit neuraminidase activity.

*C. butyricum* CB1002 did not show neuraminidase activity

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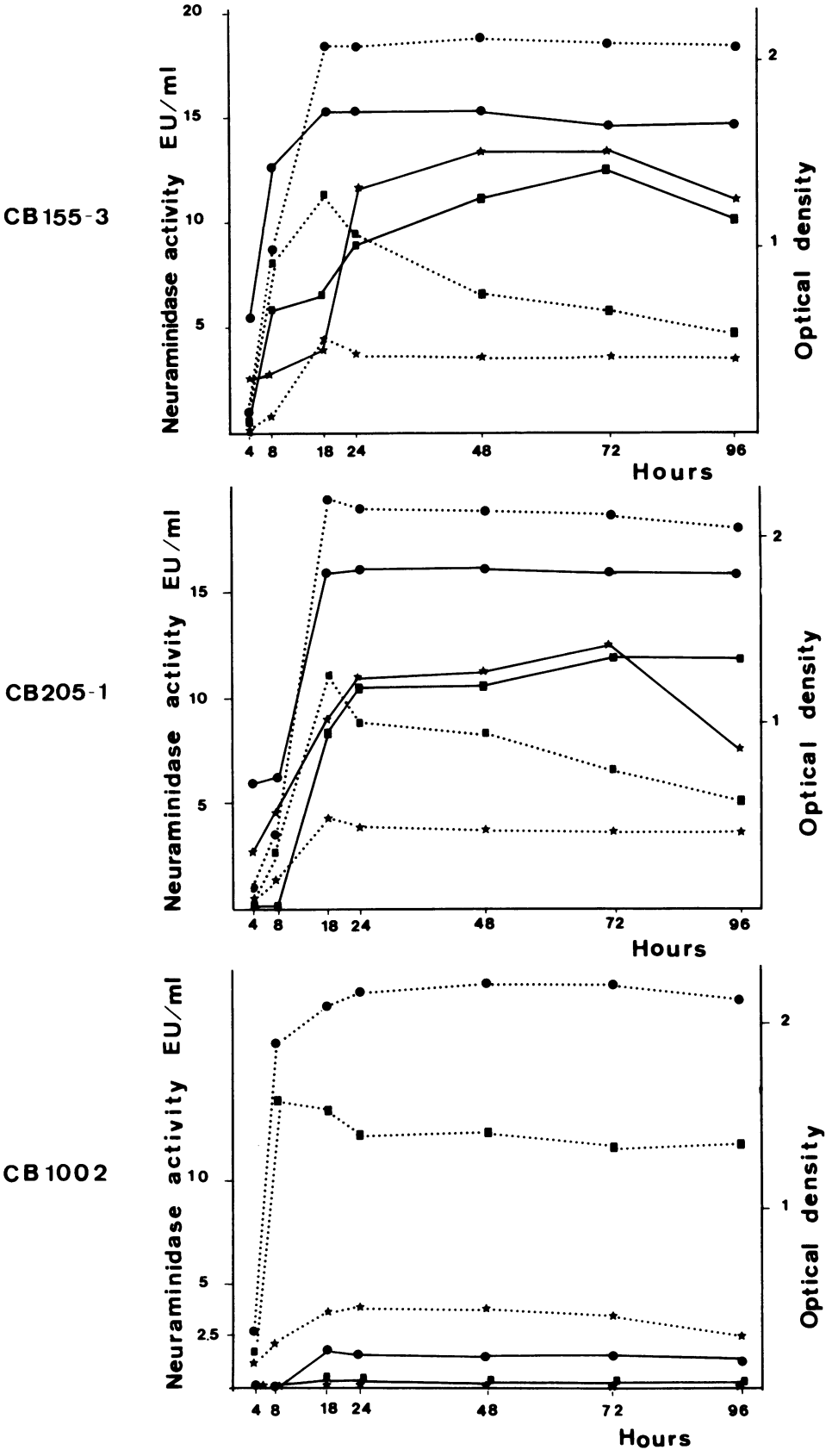


FIG. 1. Neuraminidase activity (——) and bacterial growth (·····) at various culture incubation times in TYG (●), peptone water (*), and D (■) media of *C. butyricum* strains CB155-3, CB205-1, and CB1002.

TABLE 1. Neuraminidase activity in *C. butyricum* and *C. beijerinckii* strains from various sources

| Strains (no. of strains) | Neuraminidase activity | Source (reference) |
|---|---|---|
| *C. butyricum* (57) | | |
| CB155-3 | + | |
| CB1001, CB1002, CB1003, CB1004, CB1005, CB1020, CB66082, CB103-1, CB116-1, CB117-1, CB120-1, CB123-1, CB128-1, CB143-1, CB161-1, CB171-2 | − | Feces of newborns with NNE (M. R. Popoff and N. Truffaut, Curr. Microbiol., in press) |
| CB37, CB48, CB49, CB267, CB269, CB309 | − | Feces of newborns, onset of NNE (14) |
| CB205-1 | + | Feces of newborns with hemorrhagic colitis (Popoff and Truffaut, in press) |
| CB206-1 | + | |
| CB87-2, CB133-1, CB140-1, CB144-1, CB144-3, CB153-2, CB147-3, CB165-2, CB177-1, CB180-2, CB181-1, CB203-1, CB211-2, CB214-1 | − | |
| CM474 | + | Infected placenta (14) |
| CB48381 | + | Peritonitis |
| CB52-2, CB145-1 | − | Feces of newborns with acute diarrhea (Popoff and Truffaut, in press) |
| CM263, CB278 | − | Newborn blood culture (14) |
| CB246, CB756 | − | Newborn gastric juice (14) |
| CB19-1, CB25-2, CB38-1, CB46-1, CB57-1, CB141-2 | − | Feces of healthy newborns (Popoff and Truffaut, in press) |
| CB56-84 | − | Dairy product |
| VPI 3266, VPI 1718, VPI 2969 | − | Virginia Polytechnic Institute and State University (6) |
| *C beijerinckii* (16) | | |
| CNRZ528, CNRZ529, CNRZ530, CNRZ531, CNRZ533, CNRZ534, CNRZ555, CNRZ653, CNRZ654 | − | Dairy products (15, 20) |
| CB90 | − | Unknown (15) |
| VPI 5481, VPI 2681, VPI 2966, VPI 2980, VPI 2983 | − | Virginia Polytechnic Institute and State University (6) |
| CB7423 | − | Unknown (collection of A. R. Prevot [Popoff and Truffaut, in press]) |

TABLE 2. Neuraminidase-negative *C. difficile* strains from newborns

| Strain | Source[a] |
|---|---|
| CD67-2, CD88-2, CD94-1, CD30-1, CD113-3, CD143-3, CD150-1, CD194-1, CD216-1 | Newborns with NNE |
| CD156-3, CD20-2, CD77-1, CD111-1, CD157-1, CD158-1, CD172-2, CD172-5 | Newborns with hemorrhagic colitis |
| CD184-1, CD196-1, CD213-1, CD35-1, CD39-1, CD40-1, CD48-1, CD64-1 | Healthy newborns |

[a] All strains were isolated from feces of newborns by P. Raibaud.

either in TYG broth or in TYG broth with 1% (wt/vol) porcine stomach mucin. Neuraminidase activity was also not detected in bacterial cell extracts.

None of the *C. difficile* strains showed neuraminidase activity (Table 2). The *C. difficile* strains were isolated from feces of newborns, including nine newborns with NNE, eight with hemorrhagic colitis, three with acute diarrhea, and five without disease.

Some *Clostridium* species are known to exhibit neuraminidase activity. Fraser and colleagues (7, 9) reported production of neuraminidase by *C. chauvoei*, *C. perfringens*, *C. septicum*, *C. sordellii*, and *C. tertium* but not by any *C. butyricum* strain. Caselitz and Stein (4) studied one *C. butyricum* strain that exhibited neuraminidase activity. Seger et al. (22) isolated another neuraminidase-producing strain from a newborn with NNE.

Brown and Sweet (3) suggested that neuraminidase could be a virulence factor induced by *Clostridium* species in NNE. The neuraminidase could act on the newborn digestive tract by participating in the degradation of the protective mucin layer (21) or by enhancing the effects of the bacterial toxins (3).

Our results show that neuraminidase is a potential virulence factor in some *C. butyricum* strains, but it is not a common factor.

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