NEURAMINIDASE PRODUCTION BY BACTEROIDACEAE

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SUMMARY. The production of neuraminidase (EC 3.2.1.18) by 77 strains of Bacteroidaceae was investigated by techniques previously used to study neuraminidase production by clostridia. Conditions for culture and assay of *Bacteroides fragilis* neuraminidase were characterised. The enzyme is predominantly cell associated; it is not calcium dependent and the pH optimum for its production is *c.* 4.5.

Most neuraminidase-positive *Bacteroides* strains produced the enzyme well in the test media but a few strains failed to produce it consistently in one or other of the media. Because of these occasional variations, strains were grown and tested in at least two media before being defined as neuraminidase negative.

Within the *B. fragilis* group of species, *B. fragilis*, *B. vulgatus*, *B. distasonis*, *B. ovatus*, *B. thetaiotaomicron* and *B. variabilis* were neuraminidase positive while *B. eggerthii*, *B. uniformis* and *B. splanchnicus* were negative. Two subspecies of *B. melaninogenicus* (ss. *melaninogenicus* and ss. *levii*) were positive but the other (ss. *intermedius*) was negative. Strains of *B. oralis* and *B. bivius* produced the enzyme while *B. ruminicola*, *B. disiens*, *B. asaccharolyticus* and *B. corrodens* did not. The microaerophilic *B. ochraceus* were also positive. None of the *Fusobacterium* or *Leptotrichia* species tested produced neuraminidase.

Our results for neuraminidase production are consistent for all strains of each species examined and we suggest that tests for neuraminidase production would be a valuable addition to biochemical tests currently used in taxonomic studies of the Bacteroidaceae.

INTRODUCTION

The importance of the *Bacteroides-Fusobacterium* group of gram-negative anaerobic bacilli as significant pathogens in various infections has become increasingly apparent with recent improvements in methods for their isolation and identification (Finegold, 1977). Little is known of their mechanisms of pathogenicity, and the biochemical attributes of the different species are still being defined.

Neuraminidases (EC 3.2.1.18) are produced by many species of aerobic and

anaerobic bacteria (see Müller, 1974). The enzyme is thought to play a significant role in infection with influenza virus and other myxoviruses (Drzeniek, 1972; Rosenberg and Schengrund, 1976) and it has been suggested that it may also be a virulence factor for some pathogenic bacteria (Collee, 1965; Müller, 1974).

Müller and Werner (1970*a* and *b*) reported production of neuraminidase by some *Bacteroides* species and argued that the enzyme had a role in pathogenesis. We have previously reported studies of production of neuraminidase by clostridia (Fraser and Collee, 1975; Fraser, 1978) and have now extended these studies to a range of well characterised strains representing the major species of gram-negative non-sporing anaerobic bacilli.

MATERIALS AND METHODS

Bacterial strains. Many of the strains were characterised in detail in this laboratory and described by Duerden, Holbrook, Collee and Watt (1976) or Deacon, Duerden and Holbrook (1978). The other strains studied were as follows: *Bacteroides eggerthii* NCTC11155, *B. splanchnicus* NCTC10825 and NCTC10826, *B. asaccharolyticus* NCTC9337 and *Fusobacterium varium* NCTC10560; these were obtained from the National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London NW9 5HT. *B. uniformis* VPI11227, *B. variabilis* VPI11368 and *B. melaninogenicus* ss. *levii* VPI3300 were obtained from Dr Lillian V. Holdeman, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24060, USA. *B. oralis* VPI8906D and VPI9958, *B. bivius* VPI5540, VPI6318, VPI6822 and VPI7880, and *B. disiens* VPI7852 and VPI8057 were provided by Dr Ella M. Barnes, Agricultural Research Council, Food Research Institute, Colney Lane, Norwich NOR 7OF. *B. ruminicola* strains B38024, B38080 and B56029 were provided by Dr T. Mitsuoka, Animal Pathology Laboratory, Institute of Physical and Chemical Research, Wako, Saitama 351, Japan. *B. corrodens* strains 143A and 151RV were provided by Dr A.L. James, Department of Chemistry, Faculty of Science and Technology, Newcastle upon Tyne Polytechnic. The GNAB and WPH strains were isolated from various sites in healthy adults in this laboratory, or from clinical material by colleagues in the diagnostic laboratories in the Bacteriology Departments of the Royal Infirmary and the Western General Hospital, Edinburgh. All strains were identified to species level by the methods described by Duerden *et al.* (1976).

Culture media and method of anaerobic culture. Cooked-meat broth (CMB), 5% proteose peptone water broth (PPW5) and Todd-Hewitt broth (THB) were prepared as described by Fraser (1978). Thioglycollate broth (TGB) was prepared according to the manufacturer's recommendations (Thioglycollate Medium, fluid, without dextrose or Eh indicator, code 11727; BBL, Becton Dickinson UK Ltd, York House, Empire Way, Wembley, Middlesex HA9 0PS) with the addition of 0.25% yeast extract (Oxoid) and 0.25% sodium succinate (BDH) to improve growth of *Bacteroides* strains. Digest broth (DB) was prepared as described by Cruickshank *et al.* (1975) but with best beef steak in place of horse flesh; the NaHCO₃ was omitted and the final pH adjusted with NaOH to 7.4–7.6 before sterilisation by autoclaving. PPY broth medium was as described by Deacon *et al.* (1978). The BM broth medium used in this study was prepared as described by Deacon *et al.* (1978) but 0.25% sodium succinate was added and horse serum omitted.

CMB was used without added growth factors but all other media were supplemented with haemin and menadione as a routine; in addition, cysteine hydrochloride and Na₂CO₃ were added to PPY and BM media. Solutions of these supplements were sterilised by filtration and added aseptically to the steamed media just before inoculation as described by Deacon *et al.* (1978). The final concentrations were: haemin, 5 µg/ml; menadione, 1 µg/ml; cysteine hydrochloride, 0.075% (w/v); and Na₂CO₃, 0.04% (w/v).

Anaerobic culture methods were those described by Fraser and Smith (1975). Cultures were prepared from lyophilised stock and maintained in CMB. For standard tests, an inoculum of 0.2 ml of a 48-h anaerobic culture in CMB incubated at 37°C was added to 10 ml of steamed medium and incubated anaerobically at 37°C for 48 h. Growth was estimated visually and recorded as + + +, + +, +, $\pm$ or - as described by Fraser (1978). Incubation was continued to 96 h to improve growth when there was < + turbidity at 48 h.

Preparation of cell extracts. The 48-h or 96-h cultures were centrifuged (800 *g* for 60 min at 4°C). If the culture supernates were to be tested, 5-ml samples of the centrifuged supernates were removed and stored at -20°C. The rest of the supernate was then removed from the packed cell deposit and the cells from the 10 ml culture were resuspended in 2 ml of sterile normal saline. The resuspended cells were subjected to ultrasonic disintegration for 15–20 min in an ice bath (Fraser and Collee, 1975) and these uncentrifuged cell extracts were stored at -20°C until assayed for neuraminidase.

A more concentrated bulk cell extract was prepared for tests of the effects of pH and Ca²⁺ on neuraminidase activity of *B. fragilis* NCTC9344. An inoculum of 2 ml of a 48-h CMB culture was added to 200 ml of PPW5 broth and incubated anaerobically at 37°C for 48 h. After centrifugation, the packed cell deposit was resuspended in 5 ml of sterile normal saline and disrupted by ultrasonic vibration; the uncentrifuged cell extract was stored at -20°C.

Assay for neuraminidase activity. Human glycoprotein substrate FVII (Fraser and Smith, 1975) was used at a final concentration of 140–170 μ g N-acetyl neuraminic acid (NANA)/ml reaction mixture (Fraser, 1978).

The standard assay procedures used by Fraser (1978) were followed. Tests for neuraminidase activity were first incubated for 60 min before assay for free NANA; when the 60-min assay showed little or no activity, tests were incubated for 24 h to demonstrate the presence or absence of detectable neuraminidase. Test spectrophotometric readings (E_{549}) were corrected by subtraction of the sum of the values for separate enzyme and substrate controls.

The corrected results of neuraminidase assays were graded as follows: + + +, high values in 60-min assays ($E_{549} > 0.5$); + +, moderate NANA release clearly demonstrable in 60-min assays ($E_{549} > 0.1$); +, neuraminidase not demonstrable in 60-min assays but high values ($E_{549} > 0.5$) in 24-h assays; $\pm$, low values ($E_{549} < 0.5$ but > 0.05) in 24-h assays and absorption peak clearly shown at 549 nm; -, no peak at 549 nm demonstrable in 24-h assays.

Tests that gave low values ($E_{549} < 0.5$) in 24-h neuraminidase assays were routinely examined to confirm that the absorption peak was at 549 nm (Fraser, 1978) and at least one test from each neuraminidase-positive species was also examined in this manner, whether the assay values were high or low. Culture products that gave very low values or negative results in 24-h neuraminidase assays ($E_{549} < 0.5$) were further examined to determine whether destruction of NANA by N-acetyl neuraminic acid-aldolase (NAN-aldolase, EC 4.1.3.3) might be interfering with the 24-h neuraminidase assay (Fraser, 1978).

Studies of the effect of pH on the neuraminidase of B. fragilis. Neuraminidase assays were performed in a range of sodium-acetate buffers as described by Fraser and Smith (1975). The reaction mixture of 1.0-ml volume consisted of: 0.1 ml of the bulk cell extract of *B. fragilis* NCTC9344 (see above) diluted 1 in 40 in distilled water; 0.65 ml of the appropriate acetate buffer; and 0.25 ml of substrate FVII diluted in distilled water to give a final concentration in the reaction mixture of 160 μ g NANA/ml. Separate enzyme and substrate controls were prepared at each pH value. Tests and controls were incubated at 37°C before the assay for NANA.

The pH values shown in the figure are the initial values for the buffers added to the tests. Separate measurements were made of the initial pH values in sample reaction mixtures prepared with equivalent proportions but in 3-ml total volumes.

Effect of Ca²⁺ and EDTA on the neuraminidase of B. fragilis. Neuraminidase assays in the presence of added Ca²⁺ or EDTA were performed as described by Fraser (1978). The test enzyme preparation was the bulk cell extract of *B. fragilis* NCTC9344 (see above) diluted 1 in 40 in acetate buffer, pH 5.1. Samples of the enzyme were mixed with buffer containing Ca²⁺ or EDTA and held at 37°C for 30 min before addition of substrate FVII and the start of the neuraminidase assay. Separate enzyme and substrate control tubes also contained Ca²⁺ or EDTA.

RESULTS

Production of neuraminidase by Bacteroides fragilis NCTC9344

Table I records the production of neuraminidase by *B. fragilis* strain NCTC9344 in 48-h cultures in various fluid media. The culture supernates contained small amounts of enzyme activity that could be detected when the incubation time of the assays was prolonged to 24 h. Cell extracts prepared from the same cultures contained larger amounts of neuraminidase that gave high assay values (E_{549}) in 60-min assays. Neither the culture supernates nor the cell extracts had significant NAN-aldolase activity that might interfere with the assay for NANA released by neuraminidase.

The organism grew adequately in all the media tested. Growth was least good in TGB and there were difficulties in centrifuging this culture because it contains a low concentration of agar. DB, PPW5 and BM broth media were chosen for further studies of neuraminidase production by *Bacteroides* species.

Effect of pH and Ca²⁺ on neuraminidase of B. fragilis NCTC9344

A more concentrated bulk cell extract was prepared from a 200-ml PPW5 culture of *B. fragilis* strain NCTC9344 for studies of the effect of pH and Ca²⁺ on the enzyme. The figure shows the results of 30-min neuraminidase assays in acetate buffers at different pH values. The assay values were corrected by subtraction of the values obtained in separate substrate and enzyme controls at each pH value; the enzyme controls were constant but the substrate controls were higher at low pH values.

The figure shows the pH optimum for *B. fragilis* neuraminidase with substrate FVII to be about 4.2. Acetate buffer at pH 5.1 is used in the standard assay (Fraser, 1978); this pH allows good neuraminidase activity and this

TABLE I
Production of neuraminidase by Bacteroides fragilis NCTC9344 in various culture media

Culture medium*	Degree of growth†	Relative amount of neuraminidase activity in culture product‡:	
		culture supernate	cell extract
DB	+++	±	++
PPW5	++	±	+++
BM	++	±	+++
CMB	+++	±	+++
PPY	++	±	+++
TGB	+	+	+++
THB	+++	±	+++

* DB = Digest broth; PPW5 = proteose peptone water; BM = BM medium; CMB = cooked-meat broth; PPY = PPY medium; TGB = thioglycollate broth; THB = Todd-Hewitt broth.

† See *Methods* for assessment of growth.

‡ Supernates of 48-h cultures tested in 24-h assays; cell extracts from the same cultures tested in 60-min assays. See *methods* for grading of neuraminidase activity

FIG.—Effect of pH on the assay for the neuraminidase of *B. fragilis*. Cell extract of *B. fragilis* strain NCTC9344 incubated with substrate FVII in sodium-acetate buffers.

buffer at this pH was therefore used in our tests for neuraminidase production by other *Bacteroidaceae*. Direct measurement of pH in equivalent test mixtures showed that the actual pH values might be 0.2–0.3 units higher, but they did not vary during incubation of the tests; thus the pH optimum for *B. fragilis* neuraminidase is likely to be about 4.5.

Table II shows that the addition of Ca^{2+} did not affect the assay for *B. fragilis* neuraminidase. The addition of 1 mM EDTA did not inhibit the enzyme and even 5 mM EDTA reduced the assay value by about one third only. Ca^{2+} was not added as a routine to our assays for neuraminidase production by *Bacteroides* strains.

Production of neuraminidase by species of the Bacteroides fragilis group

Table III shows the results of measuring neuraminidase produced by various species of the *B. fragilis* group. The strains were grown for 48 h in DB and PPW5 broths and cell extracts were tested for neuraminidase activity. In general these organisms grew better in DB than PPW5 broth but neuraminidase-positive strains usually produced greater activity in PPW5. All the strains of *B. fragilis* and *B. vulgatus* produced large amounts of neuraminidase in both media, and the strains of *B. distasonis* and *B. ovatus* were also clearly positive.

TABLE II
Effect of Ca²⁺ and EDTA on the assay of neuraminidase from B. fragilis NCTC9344

Concentration of added Ca ²⁺ or EDTA in reaction mixture*	Result of neuraminidase assay (E ₅₄₉)†
3mM Ca ²⁺	0.186
1mM Ca ²⁺	0.171
None added	0.188
1mM EDTA	0.171
5mM EDTA	0.118

* See *Methods* for reaction mixtures.

† The spectrophotometric value (E₅₄₉) is an average result obtained from duplicate 15-min assay readings corrected by subtraction of the values for the corresponding substrate and enzyme controls.

All strains of *B. thetaiotaomicron* produced neuraminidase well in PPW5 but production in DB varied. Strain NCTC10582 was cultured in DB on four occasions; neuraminidase assays were twice positive (+ and ++) and twice negative. Variable results were also obtained with strain GNAB16 which was once positive (+) and twice negative. By contrast, three separate cultures of each organism in PPW5 broth were consistently positive.

Results with *B. variabilis* also varied. Strain GNAB18 consistently produced neuraminidase. Strain VPI11368 twice produced moderate amounts (++) in PPW5 but failed, however, to produce detectable activity in one other PPW5 culture and in three cultures in DB medium.

All the strains of *B. uniformis*, *B. eggerthii* and *B. splanchnicus* grew adequately but did not produce detectable neuraminidase in either medium.

Neuraminidase production by other Bacteroides species

The results of tests with other *Bacteroides* species are shown in table IV. All strains grew adequately in DB and most also grew well in BM medium. Many of these strains grew very poorly or not at all in PPW5 broth and so were not tested for neuraminidase production in this medium.

The three strains of *B. melaninogenicus* ss. *melaninogenicus* and the single strain of *B. melaninogenicus* ss. *levii* produced neuraminidase. Production by strain WPH67 varied; it twice gave ++ activity in DB medium but was negative on another occasion and was twice negative in BM medium. The other strains were consistently positive in both media. By contrast the five strains of *B. melaninogenicus* ss. *intermedius* were negative in both media.

The strains of *B. oralis* and *B. bivius* were neuraminidase positive while *B. ruminicola*, *B. disiens*, *B. asaccharolyticus* and *B. corrodens* were all negative in both media. The *B. corrodens* strains did not grow well in BM broth; they were also grown and found to be negative in PPW5 medium.

Four strains of *B. ochraceus* produced neuraminidase well in DB, but production was very unreliable in BM and PPW5 media. Strain 79B produced trace amounts on three occasions in DB and once in BM broth. Strain VPI2845 produced a trace of activity in only one of three cultures in DB and

TABLE III

Production of neuraminidase by organisms of the Bacteroides fragilis group grown in broth media

Test organism: strain no.	Growth* in		Relative amount of neuraminidase activity in culture product†	
	DB	PPW5	DB	PPW5
<i>B. fragilis</i>				
NCTC9343	+++	+++	+	+++
NCTC9344	+++	+++	+++	+++
WPH1	+++	+++	+++	+++
WPH12	+++	+++	+++	+++
WPH21	+++	+++	+++	+++
<i>B. vulgatus</i>				
NCTC10583	+++	+++	+++	+++
GNAB9	+++	+++	++	+++
GNAB29	+++	+++	++	+++
<i>B. distasonis</i>				
ATCC8503	+	++	+	++
GNAB26	++	+	++	+++
GNAB39	++	+	++	+++
<i>B. ovatus</i>				
ATCC8483	+++	+++	+	++
WPH207	+++	+++	±	++
<i>B. thetaiotaomicron</i>				
NCTC10582	+++	++	V	+++‡
GNAB1	+++	++	++	+++
GNAB2	+++	++	++	+++
GNAB7	+++	++	±	+++
GNAB11	+++	++	++	+++
GNAB16	++	+	V	++‡
GNAB20	+++	++	++	+++
<i>B. variabilis</i>				
VPI11368	+++	++	-‡	V
GNAB18	+++	++	++‡	+++‡
<i>B. uniformis</i>				
ATCC8492	+++	+++	-	-
VPI11227	+++	++	-	-
GNAB42	+++	+++	-	-
<i>B. eggerthii</i>				
NCTC11155	++	+	-	-
GNAB21	+++	+	-	-
GNAB23	+++	++	-	-
GNAB24	++	+	-	-
GNAB41	++	+	-	-
GNAB43	+++	++	-	-
<i>B. splanchnicus</i>				
NCTC10825	+++	+++	-	-
NCTC10826	+++	+++	-	-

* See *Methods* for assessment of growth. DB=Digest broth; PPW5=Proteose peptone water.† Cell extract from 48-h culture in stated medium was tested. See *Methods* for grading of neuraminidase activity. V=variable results in different experiments (see text).

‡ Result confirmed in repeat culture.

TABLE IV
Production of neuraminidase by strains of various *Bacteroides* species grown in broth media

Test organism: strain no.	Growth* in		Relative amount of neuraminidase activity in culture product†	
	DB	BM	DB	BM
<i>B. melaninogenicus</i> <i>ss. melaninogenicus</i> ATCC15930	++	++	++	+++
VPI4196	++	+++	±	++
WPH67	+++	+++	V	—*
<i>B. melaninogenicus</i> <i>ss. levii</i> VPI3300	+++	+++	+++	+++
<i>B. melaninogenicus</i> <i>ss. intermedius</i> NCTC9336	+++	++	—	—
NCTC9338	++	+	—	—
WPH4	+++	+++	—	—
WPH26	+++	++	—	—
WPH31	++	++	—	—
<i>B. oralis</i> VPI8906D	++	++	++	++
VPI9958	++	...	+++	...
GNAB46	++	...	+	...
GNAB54	+	...	+	...
<i>B. bivius</i> VPI5540	+	++	+	++
VPI6318	+	...	+	...
VPI6822	++	...	++	...
VPI7880	++	...	++	...
<i>B. ruminicola</i> NP333	++	++	—	—
B38024	+++	+	—	—
B38080	+	+	—	—
B56029	+	+	—	—
<i>B. disiens</i> VPI7852	+	+++	—	—
VPI8057	+++	+++	—	—
<i>B. asaccharolyticus</i> NCTC9337	++	++	—	—
WPH15	+	++	—	—
WPH30	++	++	—	—
WPH57	++	+	—	—
<i>B. corrodens</i> 143A	+	±	—	—
151RV	+	±	—	—
<i>B. ochraceus</i> VPI2845	++	+	V	—‡
10	++	+	++	—
73	++	++	++	±
79B	+++	+	±‡	V
1956C	+++	+	+++	—
2467B	+++	++	++	—

* See *Methods* for assessment of growth. . . = not tested; DB = digest broth; BM = BM broth.

† Cell extract from culture in stated medium was tested. See *Methods* for grading of neuraminidase activity; . . . = not tested. V = variable results in different experiments (see text).

‡ Result confirmed in repeat culture.

was consistently negative in BM and PPW5 cultures. The difficulty in demonstrating neuraminidase activity in these cultures may be partly explained by the presence of small amounts of NANA-destroying activity in the cell extract of several of the *B. ochraceus* cultures.

Production of neuraminidase by Fusobacterium and Leptotrichia species

Table V shows the results when eight strains of *Fusobacterium* species and one of *Leptotrichia buccalis* were grown in DB and PPW5 media. In general they grew well in DB and less well in PPW5. The two strains that grew poorly in PPW5 were also grown and tested in BM medium. Cell extracts of all cultures were neuraminidase negative.

TABLE V

Tests for production of neuraminidase by Fusobacterium and Leptotrichia species grown in broth media

Test organism: strain no.	Growth* in		Relative amount of neuraminidase activity in culture product†	
	DB	PPW5	DB	PPW5
<i>F. necrophorum</i>				
NCTC10575	+++	++	—	—
NCTC10576	++	+	—	—
NCTC10577	++	+	—	—
<i>F. polymorphum</i> (<i>F. nucleatum</i>)				
NCTC10562	+++	+	—	—
WPH142	++	+	—	—
WPH160	++	±	—	...
<i>F. necrogenes</i>				
NCTC10723	+	+	—	—
<i>F. varium</i>				
NCTC10560	++	+	—	—
<i>L. buccalis</i>				
NCTC10249	++	—	—	...

* See *Methods* for assessment of growth. DB = digest broth; PPW5 = proteose peptone water.

† Cell extract from culture in stated medium was tested. See *Methods* for grading of neuraminidase activity. ... = Not tested.

DISCUSSION

Neuraminidase is produced as an extracellular enzyme in culture supernates by *Vibrio cholerae* and *Clostridium perfringens*, but is cell-associated in other species, e.g., *Klebsiella aerogenes* and *Pasteurella multocida* (see Rosenberg and Schengrund, 1976). Our previous studies confirmed that clostridial neuraminidases are essentially extracellular, although some more enzyme could be released by ultrasonic disintegration of the cells (Fraser and Collee, 1975; Fraser, 1978). By contrast, the results presented in table I show that the

neuraminidase of *B. fragilis* is predominantly cell associated, with only small amounts detectable in the supernates of 48-h cultures.

We have adapted our clostridial neuraminidase-assay techniques for use with cell extracts of *B. fragilis* and other Bacteroidaceae. The pH optimum for neuraminidases may vary considerably with different buffer systems and different substrates (Fraser and Smith, 1975; Rosenberg and Schengrund, 1976) but our standard assay with human glycoprotein substrate FVII with acetate buffer at pH 5.1 also gave good results with the *B. fragilis* enzyme (see the figure). The neuraminidase produced by certain species, e.g., *V. cholerae*, is calcium dependent but added Ca^{2+} is not required in our assays for the enzyme produced by clostridia (Fraser, 1978) or *Bacteroides* species (table II).

Many of the Bacteroidaceae are nutritionally fastidious and do not generally grow well in fluid media even with growth supplements and meticulous anaerobic technique. The three media used in the present study were chosen for their ability to support good enzyme production by neuraminidase-positive species and also to allow good growth of a wide range of gram-negative anaerobes. DB medium allowed adequate growth of all species tested but did not always allow the best production of neuraminidase. Some of the *B. fragilis* group of organisms produced more enzyme in PPW5 broth; however, this medium did not support adequate growth of several other species, even when incubation was extended to 96 h. A few of these strains produced more enzyme in BM broth than in DB medium, but there were also clear exceptions. Neuraminidase-positive strains generally produced the enzyme in each of the two media in which they were grown, but production by a few varied or did not take place in one or other (see tables III and IV). The purity of all cultures was carefully tested by subculture from the test culture. Neuraminidase production has been shown to be inducible in certain species (Nees and Schauer, 1974; Rosenberg and Schengrund, 1976) but we have not been able to identify specific inducing conditions that might explain the variations observed in this study between different media or different batches of the same medium.

Because no single medium consistently supported optimum production by neuraminidase-positive strains, other strains were tested in at least two media that supported adequate growth. All cell extracts were tested in assays incubated for 24 h before being classed as neuraminidase negative.

The neuraminidase assay depends upon detection of NANA released from the glycoprotein substrate by the enzyme. Some organisms also produce an aldolase (NAN-aldolase) that may destroy the released NANA and this might give false negative results in neuraminidase assays. The NAN-aldolase of clostridia remains cell associated and does not interfere with assays for neuraminidase in culture supernates, although it may reduce the sensitivity of assays with cell extracts (Fraser and Collee, 1975). Müller and Werner (1970*b*), using a paper chromatographic method, reported the presence of NAN-aldolase in several species of *Bacteroides*, including *B. fragilis*, and Müller (1973) also found that a strain of *Fusobacterium polymorphum* produced the aldolase. We have not assayed directly for NAN-aldolase (Brunetti, Swanson and Roseman, 1963; Nees *et al.*, 1976) but we have shown that there is negligible NANA-

destroying activity in the cell extracts of the neuraminidase-positive *B. fragilis* NCTC9344 and in the cell extracts of the various cultures that gave low values or negative results in neuraminidase assays. Very few of these cell extracts reduced the assay value for added NANA by as much as 10–20% during 24-h incubation under conditions equivalent to those of the neuraminidase assay. This amount of NANA destruction would not appreciably reduce the ability of the assay to detect small amounts of neuraminidase. Several of the *B. ochraceus* cell extracts reduced the added NANA by about 40% in 24 h and this could have contributed to our difficulty in demonstrating neuraminidase production consistently in this group.

The classification of the Bacteroidaceae has been much refined in the past decade. Current schemes of classification are based mainly on the biochemical activities of the organisms (Holdeman and Moore, 1974; Holdeman, Cato and Moore, 1977). The strains used in the present study have been characterised carefully by the methods used in this laboratory, including gas–liquid chromatography (Duerden *et al.*, 1976; Deacon *et al.*, 1978; Duerden *et al.*, 1980). The former subspecies of *B. fragilis* are now given separate species status, and *B. thetaiotaomicron* is further subdivided into the species *B. thetaiotaomicron*, *B. eggerthii*, and *B. uniformis*. Strain ATCC8492, originally the reference strain of *B. thetaiotaomicron*, is now classified as *B. uniformis*. Werner (1974) demonstrated that sugar fermentation tests discriminated reliably between the various species in this group; he further distinguished *B. variabilis* from *B. uniformis* by differences in fermentation of rhamnose and trehalose.

The classification of the *B. oralis*–*B. melaninogenicus* group has been considerably revised in recent years, with less emphasis placed on pigment production as the criterion for classifying strains as *B. melaninogenicus*. Because of the basic difference in sugar utilisation, *B. melaninogenicus* ss. *asaccharolyticus* has been given separate species status as *B. asaccharolyticus*, while *B. melaninogenicus* is now divided into the subspecies *melaninogenicus*, *levii* and *intermedius*. There has been some debate about the classification of *B. oralis* strains. Strain ATCC15930, originally a reference strain of *B. oralis*, is now reclassified as *B. melaninogenicus* ss. *melaninogenicus* (Holbrook and Duerden, 1974). Strain NP333, previously described as *B. oralis*, is now reallocated to *B. ruminicola*. Strains of *B. ochraceus* differ from other Bacteroidaceae in that they require CO₂ for growth but are not strict anaerobes (Holbrook, Duerden and Deacon, 1977). It has recently been proposed that they should be reclassified in the new genus *Capnocytophaga* (Newman *et al.*, 1979; Williams, Hollis and Holdeman, 1979).

Our results for neuraminidase production by *Bacteroides* are consistent within each species and tend to confirm the present classification. Within the *B. fragilis* group, *B. fragilis*, *B. vulgatus*, *B. distasonis* and *B. ovatus* were all neuraminidase positive. Seven strains of *B. thetaiotaomicron* were positive while six strains of the closely related *B. eggerthii* were negative. The *B. variabilis* strains were distinguished from the *B. uniformis* strains by their production of the enzyme. Within the *B. oralis*–*B. melaninogenicus* group, *B. oralis* and *B. melaninogenicus* ss. *melaninogenicus* were neuraminidase positive

while ss. *intermedius* and *B. ruminicola* were negative; the single strain of *B. melaninogenicus* ss. *levii* tested was neuraminidase positive. *B. bivius* produced the enzyme, while the closely related *B. disiens* did not. The asaccharolytic species, *B. asaccharolyticus* and *B. corrodens*, were both negative. The six strains of *B. ochraceus* tested could all produce neuraminidase although it was difficult to define conditions allowing reliable production.

Production of neuraminidase by *Bacteroides* species was studied by Müller and Werner (1970*b*; Werner and Müller, 1971). These studies depended primarily on observing the electrophoretic changes in serum proteins in the culture medium after incubation for 2–19 days, but were also supported by paper chromatography of neuramin-lactose and its split products. They found that all of 24 strains of *B. fragilis* produced the enzyme, but only six of 11 *B. vulgatus*, three of five *B. distasonis* and six of eight *B. thetaiotaomicron*. With our assay procedures, testing cell extracts of the cultures, all the strains of these species examined were neuraminidase positive. At least one of their negative *B. thetaiotaomicron* strains (ATCC8492) is now reallocated to *B. uniformis*; this strain was negative in our assays also. Their findings with other *Bacteroides* species are in broad agreement with our present results: *B. ovatus* ATCC8483, three strains of *B. oralis* (including ATCC15930) and one strain of *Leptotrichia innominata* var. *ochracea* (*B. ochraceus*) were positive, and single strains of *B. asaccharolyticus* (NCTC9337) and of *B. putredinis* were negative.

Müller and Werner (1970*a*) also studied pus from a *B. fragilis* abscess and showed that neuraminidase was produced *in vivo*. They suggested that the neuraminidase might play a role in the pathogenicity of *B. fragilis*, demonstrating that it is produced in greater amounts and has a broader spectrum of action against serum glycoproteins than the enzyme produced by other *Bacteroides* species. *B. fragilis* is now an accepted pathogen; although it is only a minor component of the anaerobic gut or vaginal flora it is much the most common *Bacteroides* species isolated from clinical infections (Duerden, 1980*a* and *b*). The pathogenic potential of other *Bacteroides* species is much less clearly understood at present; it will be interesting to note whether there is any correlation with their ability to produce neuraminidase.

Fukui, Fukui and Moriyama (1971) failed to detect neuraminidase in four strains of *Fusobacterium* species and two of *Leptotrichia buccalis* isolated from the mouth, nor could we detect the enzyme in cell extracts of the strains that we examined. However, Werner and Müller (1971) detected weak neuraminidase activity against plasma proteins in two of 10 strains of *Sphaerophorus necrophorum* (*F. necrophorum*), two of three *S. varium* (*F. varium*) including NCTC10560, and one of seven other *Fusobacterium* strains. Müller (1973) isolated a strain of *F. polymorphum* from the throat of a patient with actinomycosis; it showed strong neuraminidase activity when first isolated but this declined to a low level during subsequent laboratory passage. Müller (1973, 1974) has argued that neuraminidase plays a role in the pathogenicity of various organisms and that in some cases its production may be induced *in vivo*. The *Fusobacterium* strains that we found to be neuraminidase negative have been maintained in laboratory culture for some years and it remains

possible that they have lost the ability to produce the enzyme during storage.

Solovev *et al.* (1972) found tests for neuraminidase production of value for studies of vibrio taxonomy and our previous studies suggested that they might also help to differentiate some closely related clostridia, e.g., *C. sordellii* and *C. bifermentans* (Fraser, 1978). The taxonomy of the fusobacteria is still being debated (see Duerden *et al.*, 1980) and our failure to demonstrate neuraminidase in any of the strains tested is unlikely to be of assistance. However, the results of our tests in *Bacteroides* species are consistent and conform with the latest classifications (Holdeman *et al.*, 1977; Duerden *et al.*, 1980). They help to discriminate between several closely related species that are otherwise distinguished only by a small number of sugar fermentation reactions, e.g., between the species previously included in *B. thetaiotaomicron*, and between various species of the *B. oralis*–*B. melaninogenicus* group. Tests for neuraminidase production would therefore be a valuable addition to the range of biochemical tests currently used in classification of *Bacteroides* organisms.

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