Characteristics of an Unusual Anaerobic Pigmented Gram-Negative Rod Isolated from Normal and Inflamed Appendices

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During our studies of the bacterial etiology of appendicitis, we often isolated a previously undescribed anaerobic gram-negative rod. This organism resembled the *Bacteroides fragilis* group because it was resistant to bile and because of its special-potency-disk pattern (resistant to vancomycin, kanamycin, and colistin), but unlike the *B. fragilis* group, this bacterium produced brown pigment on media containing hemolysed blood. The cellular fatty acid pattern, with iso-C15:0 being the predominant acid, was most closely related to the fatty acid profile of *Porphyromonas* species; however, this organism differed from *Porphyromonas* species by being bile-resistant and by not producing butyrate as a metabolic endproduct. Enzymatic activities of 31 isolates were determined with use of the API ZYM system and Rosco diagnostic tablets. These profiles were different from those of *Prevotella, Porphyromonas,* and related species. This organism was isolated from 40% of appendiceal tissue samples; no obvious qualitative or quantitative difference in rates of isolation from patients with inflamed or normal appendices was observed.

Acute appendicitis is the most common surgical emergency in childhood. The etiology and pathogenesis of this condition is obscure, and various aerobic as well as anaerobic bacteria have been isolated from inflamed appendices. Bennion and colleagues [1] found an average of 11.6 organisms per specimen (an average of 8.5 anaerobes per specimen) in cultures of perforated and gangrenous appendices and peritoneal fluid or pus from adult patients. During our studies on the bacterial etiology of appendicitis in children, we often isolated from both inflamed and noninflamed appendiceal tissue a previously undescribed anaerobic gram-negative rod. Other studies on fecal flora composition also have revealed strains that did not fit any existing species designations [2, 3], but none of these were similar to the organism we describe here.

The aim of this study was to phenotypically characterize this bile-resistant, pigment-producing organism in order to devise a scheme to facilitate its recognition and identification as well as to determine its occurrence in appendiceal tissue in relation to the inflammation.

Materials and Methods

The material for this study was collected from the children's ward of Aurora Hospital, in Helsinki, where the patients were admitted for suspected appendicitis as described previously [4]. The ages of the patients ranged from 1 to 15 years (mean, 11 years), and they were grouped according to the histopathologic and clinical findings as having an inflamed or noninflamed appendix. The final diagnosis in six cases was other than appendicitis.

Altogether, 31 isolates of this bile-resistant, pigment-producing organism from 25 patients were available as frozen stock cultures for detailed bacteriologic characterization. The organism was most often isolated on bacteroides-bile-esculin (BBE) agar and less often on kanamycin/vancomycin laked blood agar or other media. The primary identification tests included those for susceptibility to special-potency antimicrobial disks (Oxoid Antimicrobial Susceptibility Test Discs; Unipath, Basingstoke, Hampshire, U.K.); catalase, indole, and nitrate reactions; and aerotolerance. The strains were further characterized with use of prereduced anaerobically sterilized (PRAS) biochemicals, gas-liquid chromatography, API ZYM (bioMérieux, Marcy l'Etoile, France) and RapID ANA II (Innovative Diagnostic Systems, Norcross, GA) panels, and Rosco diagnostic tablets (Rosco, Taastrup, Denmark) [5–7].

Bile resistance was confirmed with use of several methods: growth on BBE agar with and without gentamicin, growth around an oxgall disk (Rosco), and growth in peptone–yeast extract–glucose containing 20% bile (for some isolates). Pigment production was studied on rabbit laked blood (RLB) agar, kanamycin/vancomycin laked blood agar (containing 5% lysed sheep blood), and brucella agar with defibrinated sheep blood. Enhancement of growth by various supplements (formate/fumarate, horse serum, bile, pyruvate, sodium bicarbonate, hemin, and tween) was tested in thioglycollate medium [5, 6].

Cellular fatty acid profiles of the isolates were determined by the Microbial Identification System (Hewlett-Packard

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Table 1. Biochemical and other characteristics of the bile-resistant unusual organism.

Characteristic	No. of isolates*
Pigment production: +	31/31
Bile: R	31/31
Special potency disks	
Vancomycin: R	31/31
Kanamycin: R	31/31
Colistin: R	31/31
Production of	
Catalase: −	31/31
Indole: +	31/31
Reduction of nitrate: −	31/31
Fermentation† of	
Arabinose: −	11/11
Cellobiose: −	8/8
Glucose: +	14/15
Lactose: +	7/7
Sucrose: −	17/19
Trehalose: −	16/16
Hydrolysis of gelatin: +	30/31
GLC: apS^M (iv,1)	29/29

NOTE. apS^M (iv,1) = succinic acid as the major product, acetic and propionic acids as minor products, and isovaleric and lactic acids sometimes produced in minor amounts; GLC = gas-liquid chromatography for metabolic end products; R = resistant; + = positive; − = negative.

* No. of isolates with indicated characteristic/total no. of isolates tested.

† Reactions were recorded only when growth appeared.

[Boise, ID] and MIDI [Newark, DE]) and the plate-based method on supplemented brain-heart infusion agar because of poor growth in peptone–yeast extract–glucose broth. Fatty acid methyl esters (FAMEs) were identified with use of the computerized database library compiled by MIDI.

Susceptibility to metronidazole was determined by the disk-diffusion method (Rosco), and β-lactamase production by means of Nitrocefin disks (AB BIODISK, Solna, Sweden).

Results

The unusual pigmented organism was cultured from 40% (17) of 42 tissue specimens, and no obvious difference in rates of isolation from patients with inflamed (14 of 36 [39%]) or noninflamed (3 of 6 [50%]) appendices was noted. In addition, no significant differences in quantities was observed when the semiquantitative method was used in culturing. However, the number of noninflamed appendices with complete culture results was only six and thus does not allow definitive conclusions regarding association of the organism with health or disease. It was always isolated in mixed culture, together with other anaerobes and aerobes.

The organisms isolated were obligately anaerobic, resistant to 20% bile, and grew without inhibition around an oxgall disk. The special-potency-disk pattern showed resistance to vancomycin (5 μg), kanamycin (1,000 μg), and colistin (10 μg). All isolates studied were indole-positive and catalase- and nitrate-negative. Gram staining showed the cells were gram-negative straight rods with rounded ends measuring 0.2 by 0.8–2.0 μm, usually occurring singly; occasionally, longer filaments were observed.

After incubation for 4 days on brucella agar, surface colonies were 0.7 mm (0.3–1.0 mm) in diameter, circular, entire, raised, grey, translucent or opaque, and β-hemolytic. On RLB agar, colonies showed light brown pigment after 4 days and a reddish or chocolate brown coloration after 10 days of incubation. Under long-wave ultraviolet (UV) light, no fluorescence was observed but colonies appeared black. Pigment production or a black appearance under UV light was not seen on brucella agar, and on kanamycin/vancomycin laked blood agar the de-

Table 2. Enzyme reactions of the isolates (n = 31), as determined with use of Rosco diagnostic tablets.

Enzyme reaction	Percentage of isolates
α-Fucosidase: +	100
N-acetyl-β-glucosaminidase: +	100
Trypsin: −	100
α-Glucosidase: +	100
β-Xylosidase: −	100
ONPG (β-galactosidase): +	100
α-Galactosidase: +	100
Esculin: +	37

NOTE. + = positive; − = negative.

Table 3. Enzyme reactions of the isolates (n = 31), as determined by means of API ZYM panels.

	No. of isolates with indicated reaction pattern		
Enzyme	Strong (3–5)*	Weak (1–2)*	Negative (0)*
Alkaline phosphatase	25	6	0
Esterase (C 4)	1	30	0
Esterase lipase (C 8)	8	23	0
Lipase (C 14)	0	0	31
Leucine arylamidase	0	0	31
Valine arylamidase	0	0	31
Cystine arylamidase	0	0	31
Trypsin	0	0	31
Chymotrypsin	3	12	16
Acid phosphatase	31	0	0
Naphthol-AS-BI-phosphohydrolase	25	5	1
α-Galactosidase	22	9	0
β-Galactosidase	31	0	0
β-Glucuronidase	0	0	31
α-Glucosidase	27	4	0
β-Glucosidase	0	0	31
N-acetyl-β-glucosaminidase	30	1	0
α-Mannosidase	0	0	31
α-Fucosidase	3	16	12

* Values denote color intensites in the API ZYM color chart.

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Table 4. Cellular fatty acid compositions of the isolates.

Cellular fatty acid	Unusual organism (n = 27)	*Bacteroides* species[†]	*Prevotella* species[†]	*Porphyromonas* species[†]
13:0 iso FAME	–	1 (0–2)	1 (0–5)	4 (1–6)
14:0 iso FAME	–	1 (0–4)	5 (0–13)	–
14:0 FAME	1 (0–3)	1 (1–2)	2 (0–9)	5 (3–6)
15:0 iso FAME	38 (28–53)	6 (6–16)	10 (4–18)	45 (33–58)
15:0 ante-iso FAME	7 (5–9)	36 (32–40)	35 (22–45)	7 (2–15)
15:0 FAME	4 (1–12)	6 (2–14)	1 (0–3)	–
16:0 iso FAME	1 (0–1)	1 (0–2)	3 (0–9)	–
16:0 FAME	8 (5–12)	9 (6–12)	9 (2–24)	9 (4–13)
17:0 iso FAME	6 (1–11)	2 (0–6)	3 (0–9)	1 (1–1)
16:0 3OH FAME	3 (2–6)	4 (2–6)	5 (1–13)	3 (1–5)
18:1 cis 9 FAME	4 (1–5)	–	1 (0–3)	1 (1–2)
Summed feature 3[‡]	2 (0–6)	–	–	–
Summed feature 11[§]	7[‖] (3–11)	15 (11–19)	11 (5–18)	15 (10–20)

NOTE. Numbers are mean percentages of the total acids; the range of values for species in the genus is given in parentheses. The compounds included are those that occurred at levels of ⩾5% in one or more of the species. FAME = fatty acid methyl ester; minus (−) and 0 denote occurrence at a level of <0.5% of the total acids.

[†] Data are compiled from [8].

[‡] Possibly 15:0 iso ALDE or an unknown having an equivalent chain length of 13.570.

[§] 17:0 iso 3OH FAME or 18:2 DMA.

[‖] Confirmed as 17:0 iso 3OH FAME by mass spectrometry.

velopment of pigment was slower and the intensity remained weaker than on RLB.

Growth was scanty on successive subcultures on different agar media (brucella, RLB, fastidious anaerobe agar, BBE) and especially in liquid PRAS media. None of the supplements tested in thioglycollate medium enhanced growth, although weak stimulation was noted with horse serum and bile. Consequently, carbohydrate fermentation was very difficult to demonstrate. On testing in PRAS biochemicals, most strains fermented glucose and lactose and liquefied gelatin (table 1).

Major amounts of succinate and minor amounts of acetate and propionate were produced as metabolic endproducts of glucose fermentation.

The enzyme profiles obtained by API ZYM and Rosco diagnostic tablets are shown in tables 2 and 3. The API ZYM reactions coincided with those of the Rosco system, except for α-fucosidase, which was usually negative or weak with API ZYM: only 10% of the isolates gave clear positive reactions, vs. 100% with the Rosco system. With RapID ANA II panels the profile number was 437304, which the database manual identified as a *Bacteroides fragilis* group isolate. These panels were used to test nine isolates, and they all gave the same profile number and a positive reaction for α-fucosidase.

The predominant cellular fatty acid detected was iso-C15:0 (13-methyltetradecanoic acid), accounting for 38% of the total fatty acids present. Other FAMEs, shown in table 4, were detected in smaller amounts. The Microbial Identification System search with the MOORE Library, version 3.9 (MIDI), as a reference suggested ''*Bacteroides* AR'' as the most likely match for some strains.

All strains were susceptible to metronidazole, and 23% (7 of 31) produced β-lactamase.

Discussion

With routine tests the organism described here superficially resembles *B. fragilis* group, except that it produces brown pigment, as do the pigmented species in the genera of *Prevotella* and *Porphyromonas.* Shah and Collins [9] have proposed that the genus *Bacteroides* be restricted to include only the *B. fragilis* group and closely related organisms. Members of the *B. fragilis* group are recognized in routine microbiological culture of clinical specimens by their ability to grow in the presence of aminoglycosides and 20% bile; thus, growth on BBE medium is characteristic of the *Bacteroides fragilis* group.

Some non–*B. fragilis* group organisms such as *Bacteroides splanchnicus, Bacteroides tectum, Mitsuokella* species, *Bilophila* species, and some fusobacteria are also bile-resistant, but all of these and the *B. fragilis* group are nonpigmented. Differentiation within the *B. fragilis* group is usually done by fermentation tests, but the organism characterized here grew so poorly in PRAS biochemicals that we initially often misidentified it as *Bacteroides eggerthii,* which ferments fewer sugars than other indole-positive strains in this group.

''*Bacteroides* AR'' is also similar to *B. eggerthii,* except that arabinose is not fermented and the cells are thinner. ''*Bacteroides* AR'' is fermentative, sucrose-negative, indole-positive, and gelatin-positive, but no information about pigment production was available in the original description, and it was reported as nonhemolytic [3].

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Cellular fatty acid composition most closely resembled that of the genus *Porphyromonas.* There is general agreement and consensus that iso-C15:0 is the major detectable fatty acid in *Porphyromonas* species [8]. However, our isolates phenotypically differ from *Porphyromonas* species by being resistant to bile and by not producing butyrate as a metabolic endproduct. Ante-iso-C15:0, iso-C15:0, iso-3-OH-C17:0, and C16:0 are the major cellular fatty acids in *Bacteroides* and *Prevotella* species.

Enzyme profiles were not similar to any published profiles of *Prevotella, Porphyromonas, Bacteroides,* or closely related species [7, 10]. The closest match was to the profile of *Prevotella melaninogenica/denticola,* except for the negative leucine arylamidase reaction (100%) and at least weakly positive chymotrypsin reaction (48%) of our isolates.

The following characteristics will help one to recognize and identify the unusual gram-negative bacillus we described: resistance to special-potency-antimicrobial identification disks, growth on BBE, brown pigment on RLB media, black colonies under UV light, positive indole test, scanty growth in PRAS media, and the demonstrated API ZYM and RapID ANA patterns.

The anaerobic, bile-resistant, pigmented gram-negative bacterium described in this study exhibits phenotypic properties distinct from any previously described species.

This bacterium also has been isolated in one case of perirectal abscess and from a brain abscess in a patient with invasive squamous cell carcinoma of the scalp with extension to the nasal cavity and orbit. In both cases this bile-resistant pigmenter was found as part of a mixed flora (the first case had 4 aerobes and 14 anaerobes [*B. fragilis* group predominated], and the second had 7 aerobes and 11 anaerobes). Furthermore, our recent studies showed that strains closely related to this unusual organism can be isolated in fecal samples from both children and adults.

Further studies, involving determination of DNA relatedness and 16S rRNA sequencing, are pending to resolve the taxonomic position of this organism.

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