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Simple Medium for the Selective Isolation of *Bacteroides* and Related Organisms, and Their Occurrence in Sewage

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A medium composed of 0.009% sodium azide, 0.07% sodium deoxycholate, and 0.0007% ethyl violet in Brain Heart Infusion Agar (Difco) and a process of incubation in an atmosphere of 90% N_2 and 10% CO_2 for the selective isolation of certain members of the intestinal bacteroides are described. The medium appears to select predominantly members of the genus *Bacteroides* and a few of the genus *Sphaerophorus*. A survey of the occurrence of these organisms in sewage and various stages of sewage treatment indicates that they survive complete sewage treatment in low numbers and that their rate of decline parallels that of the coliforms. Large numbers were recovered from sludge digestion tanks, suggesting a possible role in the anaerobic breakdown of organic matter.

Because they are easily isolated and are abundant in human and animal feces, members of the coliform group of bacteria are the most commonly used indicators of sewage pollution. However, reliance on their presence has been criticized on several grounds. Coliform bacteria are not limited to humans, and thus distinction between strictly human and animal pollution is not satisfactory. Also, they occur and grow in nature in a number of places outside the intestinal tract. Considerable effort has therefore been made to find more suitable indicators.

A number of reports (3, 7, 12, 13, 14) have appeared on a group of bacteria occurring in the intestinal tract of man and animals in numbers 10 to 100 times those of the coliform group. These organisms are obligately anaerobic, gram-negative, nonsporulating rods, referred to collectively as bacteroides, and placed in the family *Bacteroidaceae* (9, 10; *Bergey's Manual of Determinative Bacteriology*). The intestinal bacteroides no doubt include a heterogenous number of species from this family, some of which may be valuable in identifying human fecal contamination. They have been little studied as a group because of their anaerobic nature and the lack of definitive selective media for isolation. Most of the media for isolation of bacteroides described in the literature (1, 2, 5, 8; S. M. Finegold, L. A. Siewert, and W. L. Hewitt, Bacteriol. Proc., p. 59, 1957) also support growth of many species of facultative and obligate anaerobes. Possibly the most selective is the antibiotic-blood-agar medium of Finegold et al. (Bacteriol. Proc., p. 59, 1957), used to isolate bacteroides from infectious processes. For routine work with water and other environmental sources, however, it was desired to have a medium without blood or antibiotics as a base. This report concerns the development of a selective medium for isolation of certain members of the intestinal bacteroides and a survey of their occurrence in feces and sewage.

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MATERIALS AND METHODS

Medium development. Agents known to be selective for gram-negative bacteria or inhibitory to gram-positive organisms were combined in various proportions and tested against members of the genus *Bacteroides* and other organisms likely to be encountered in feces, sewage, and water. Selective agents included sodium azide, sodium deoxycholate, bile, and ethyl violet. Agar was added to various combinations of ingredients, and the medium was sterilized in an autoclave. Plates were poured and inoculated after solidification. During the testing phase, test cultures were streaked on the selective medium and on a control plate without inhibitors. During later phases, dilutions of feces, water, and sewage were made in sterile screw-capped tubes containing saline buffer or Brain Heart Infusion (BHI; Difco). BHI was found to be more satisfactory as a diluent than saline and was used for the major part of the study. Preliminary recovery tests indicated that bacteroides colonies were larger and more numerous when on the surface of agar. Plates were inoculated by pipetting 0.1 ml of the dilution samples on the surface of solidified agar

plates and then spreading them with a sterile bent glass rod. Exposure to air of dilution blanks, agar plates, and samples was minimized since oxygen is toxic to many of these anaerobes.

Incubation. Plates were promptly placed in an anaerobic jar, which was evacuated to 700 mm of Hg and filled with N_2 gas twice. After a third evacuation, an atmosphere of 90% N_2 and 10% CO_2 was established. The 10% CO_2 atmosphere permitted much better growth of *Bacteroides* than N_2 alone. An anaerobiasis indicator composed of a freshly boiled solution of 6 ml of 0.1 N NaOH diluted to 100 ml, 6% glucose, and 0.015% methylene blue was included in the jar. Jars were incubated at 37 C for 4 to 5 days. Control plates were incubated both aerobically and anaerobically.

Isolation and biochemical tests. Isolates were purified by streaking onto BHI Agar, followed by anaerobic incubation. Stock cultures were maintained in Thioglycollate Broth without Dextrose (Difco) or BHI. Stock or diagnostic media were added to screw-capped tubes filled to the top. Inoculation of freshly boiled and cooled tubes was made with a long loop or capillary pipette to the bottom of the tube and the cap was tightened. There was only an occasional failure to establish anaerobic conditions by this technique.

Organisms. Bacteroides strains were isolated from human feces and were also obtained as pure cultures from S. M. Finegold, Sawtelle Veterans Hospital, Los Angeles, who also kindly verified the morphology of some of the isolates from the selective medium. Other pure cultures of test organisms were obtained from the laboratory stock culture collection and were maintained on Trypticase Soy Agar (TSA; BBL) or BHI Agar under appropriate conditions.

Coliforms. Coliforms were determined in a few samples for comparative purposes. Counts were made by use of pour plates with Violet Red Bile Agar (Difco).

RESULTS

Selective medium. A number of combinations of selective agents were tested using anaerobic and facultatively anaerobic bacterial species. These included *Escherichia coli*, *Aerobacter aerogenes*, *Clostridium sporogenes*, *C. botulinum*, *C. perfringens*, *Staphylococcus aureus*, *Salmonella typhosa*, *S. typhimurium*, *Shigella* sp., *Streptococcus faecalis*, Viridans group, *Alcaligenes faecalis*, and *Bacteroides* spp. A medium composed of sodium azide 0.009%, sodium deoxycholate 0.07%, and ethyl violet 0.0007% in BHI Agar base permitted optimal growth of the *Bacteroides* spp. and completely suppressed the other organisms under anaerobic conditions. Faint growth appeared on plates with aerobic incubation when a heavy inoculum was used, especially with the gram-positive cocci. Recovery of *Bacteroides* on this medium was later shown to be improved by the addition of serum containing laked red blood cells, but the growth of other bacteria presented a much greater problem. The final medium did not contain blood.

The selective medium was first tested qualitatively by emulsifying fecal material in 0.85% saline and streaking a portion of this on the medium. After 4 to 5 days of incubation, colonies were isolated and streaked on two BHI Agar plates; one was incubated aerobically and the other anaerobically. Occasional colonies appeared on the aerobic plates, indicating that facultative anaerobes survived on the selective medium. Further purification of the colony from the anaerobic plate demonstrated the presence of obligately anaerobic bacteroides. Isolated cultures were also tested for catalase production and were Gram-strained.

Colony types. The selective medium was next tested more extensively with feces, various stages of sewage treatment, and a few other sources. Three basic types of colonies were observed on the selective medium: small, deep blue, 1 to 2 mm in diameter, round, convex, and smooth in appearance; light blue, 2 to 5 mm diameter, varying from convex to umbonate, with a darker center and light edge; and white opaque, 3 to 5 mm diameter, flat with a rough edge, and decolorization of the medium around the colony. Isolates from 105 of these colony types were gram-negative, obligately anaerobic, nonsporeforming rods, and on the basis of morphology were placed into one of two groups (11): a nonpleomorphic *Bacteroides* group encountered in 34 of 36 deep-blue colonies, 27 of 48 light-blue colonies, and 16 of 21 white colonies, composing 73% of the colonies studied, or a highly pleomorphic *Sphaerophorus* group encountered in the other 27% of the colonies. The *Bacteroides* group included cells 1 to 2 μ long by 0.5 to 1 μ wide, with rounded ends, nonpleomorphic, occurring singly, in pairs, and occasionally in short chains, and staining evenly or bipolarly. Some strains exhibited a number of ghost cells. The *Sphaerophorus* group included highly pleomorphic cells with globular forms and the appearance of branching, with filaments 0.5 to 1 μ wide by 1 to 3 μ long usually, some being 10 to 12 μ and an occasional one 60 μ. A few isolates exhibited a marked preponderance of 10- to 60-μ filaments. Staining was bipolar and uneven. No further study of the morphologically different types was made.

Physiological studies. For more definitive studies, 570 of the various colony types were picked, and their cellular morphology and Gram reaction were observed. Six of these were obligately anaerobic, gram-negative cocci tentatively identified as *Veillonella* sp. These could not be separated from contaminating organisms, a problem observed by others (11). One gram-posi-

TABLE 1. *Summary of characteristics of 99 strains of bacteroides*[a]

Characteristics	Group A (26)				Group B (21)					Group C (21)	Group D (12)	Group E (19)				Total (99)
	22[b]	2	1	1	2	11	1	3	4	21	12	13	3	1	2	
Indole produced	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	
Gelatin liquification	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	
NO_2^- from NO_3^-	−	−	−	+	+	+	+	+	+	−	−	−	−	−	−	
Lactose, reaction with	−	G	ND	−	−	a	ND	−	−	G	a	−	−	−	ND	
Glucose, reaction with	G	G	ND	a	G	a	ND	−	a	G	a	G	a	−	ND	
Broth base, reaction with	−	−	G	−	−	−	G	−	−	−	−	−	−	−	G	
No. hemolytic	1					1	1				2	1				6
Source																
Feces				1		11		3	4		12		3	1		35
Digesting sludge	6	1	1									5				13
Other sewage	16	1			2		1			21		8			2	51
Morphology																
Bacteroides-like	19	2	1	1	1	10	1	3	4	7	10	9	3	1	2	74
Sphaerophorus-like	3				1	1				14	2	4				25

[a] Plus sign = positive reaction; minus sign = no reaction but growth occurred; G = production of acid and gas; a = production of acid only; ND = not determined.

[b] Refers to number in each subgroup.

tive rod was isolated and tentatively placed with the lactobacilli. *Bacteroides* were found in 99% (563) of the colonies. Nine of the 563 colonies were motile strains not readily separated from contaminating bacteroides and were not further studied. Of the remaining 554 colonies, 99 were randomly selected and purified, and further studies were made. A summary of the characteristics of these organisms is presented in Table 1.

Organisms were separated into groups based on arbitrarily selected physiological properties. Group A produced indole; B formed nitrite from nitrate but no indole; C produced acid and gas in glucose and lactose and was negative in other characteristics; D produced only acid from these carbohydrates; and E underwent miscellaneous reactions. Reaction of four strains on glucose and lactose could not be determined by the techniques of this study, since acid and gas were produced in small amounts in the Phenol Red Broth Base (Difco) control without added carbohydrate, a characteristic reported for some bacteroides (*Bergey's Manual of Determinative Bacteriology*). Subdivision of these groups was made on the basis of common characteristics. Several characteristics were held in common by all strains: none grew in Nutrient Broth (Difco), all grew in BHI, all were catalase-negative, and none was motile.

TABLE 2. *Summary of bacteroides and coliform plate counts of fecal samples from four individuals*

Sample	Subject	*Bacteroides* per g × 10^4	Coliforms per g × 10^4	*Bacteroides*/ coliforms
1	A[a]	13	38	.34
2	A	25	26	.96
3	A	13	410	.03
4	A	37	87	.43
5	A	18	380	.05
6	B	370	69,000	.01
7	C	96	340	.28
8	D	85	400	.21

[a] Samples from subject A taken every other day.

Results of fecal isolations from four individuals are in Table 2. The recovery ratio of bacteroides to coliforms ranged from 0.01 to 0.96 with a median value between 0.21 and 0.28. Samples were also collected of soil (5), potato salad (1), macaroni salad (1), and egg salad sandwiches (2). In most of these, bacteroides or coliform counts were below the sensitivity of the method

(i.e., 100/g). One of the egg salad sandwiches contained 900 bacteroides and 540,000 coliforms per g for a ratio of 0.0017. This portion of the study was not carried further but does warrant future investigation.

Several sewage treatment plants in the local area were sampled, including a training model on campus. The bacteroides recovery in various stages of treatment at four plants is shown in Table 3. The percentages of recovery of bacteroides and coliforms for different stages of treatment appear in Table 4. In spite of great differences in initial numbers, coliforms and bacteroides decline at similar rates as sewage passes through the treatment plant.

TABLE 3. *Bacteroides and coliform counts from various stages of sewage treatment*

Plant[a]	Treatment stage	Bacteria per milliliter	
		Bacteroides	Coliforms × 10^4
A	Raw	2,200	
	Activated sludge return	400	
	Secondary clarified	20	
	Algae pond effluent[b]	<10	
A	Raw	1,000	
	Primary clarified	340	
	Primary sludge	2,700	
	Activated sludge	520	
	Algae pond effluent[b]	220	
A	Raw	600	
	Primary clarified	550	
	Activated sludge	290	
	Secondary clarified	<10	
	Algae pond effluent[b]	10	
A[c]	Raw	1,500	
	Primary clarified	1,200	
	Primary sludge	10,000	
	Activated sludge	8,500	
	Secondary clarified	3,800	
A	Raw	1,100	23
	Primary clarified	910	70
	Activated sludge return	920	1.9
	Activated sludge	60	8.5
	Secondary clarified	<10	0.6
B	Raw	1,400	
	Activated sludge	160	
	Secondary clarified	60	
	Settled activated sludge	80	
	Digesting sludge	1,600	
B	Raw	7,700	
	Primary clarified	4,000	
	Activated sludge	2,800	
	Secondary clarified	280	
	Digesting sludge	2,600	
C	Raw	20,000	
	Primary clarified	2,000	
	Digesting sludge	26,000	
C	Raw	10,000	
	Primary clarified	2,500	
C[c]	Raw	8,500	
	Primary clarified	9,900	
	Digesting sludge	9,900	
C[c]	Raw	4,900	
	Primary clarified	5,000	
D	Raw	3,800	120
	Primary clarified	8,400	57
	Activated sludge	880	5.6
	Activated sludge return	2,400	5.5
	Secondary clarified	10	0.03
D	Raw	10,000	57
	Primary clarified	6,200	56
	Activated sludge	1,000	5.6
	Activated sludge return	3,800	27
	Secondary clarified	10	0.03

[a] A = campus, B = Hyperion (Los Angeles City), C = San Pedro (Los Angeles County), and D = valley (Los Angeles City).

[b] Algae ponds were being investigated as a form of tertiary treatment and received the effluent from the secondary clarifier.

[c] Treatment process was not functioning properly and raw sewage passed through system.

DISCUSSION

Organisms isolated from the rather distinctive colonies on the selective medium fit the general description of the family *Bacteroidaceae*. All were gram-negative, obligately anaerobic, nonsporulating rods. Classification in this group is not clear at present and ranges from the simplified groupings of a few genera (11) to the more complex groupings of Prévot (9) and others (10) on which *Bergey's Manual of Determinative Bacteriology* is based. Since this investigation was not designed to classify isolates beyond genus, a task which would require special techniques (10), isolates were placed morphologically into a

TABLE 4. *Percentage recovery of bacteroides and coliforms from successive stages of sewage treatment*

Plant	Treatment stage	Per cent bacteroides recovery [a]	Per cent coliform recovery[a]
A	Raw	100	
	Primary clarified	92	
	Activated sludge	49	
	Secondary clarified	<1	
A	Raw	100	100
	Primary clarified	82	300
	Activated sludge	6	37
	Secondary clarified	<1	3
B	Raw	100	
	Primary clarified	52	
	Activated sludge	36	
	Secondary clarified	4	
D	Raw	100	100
	Primary clarified	220	47
	Activated sludge	23	5
	Secondary clarified	<1	<1
D	Raw	100	100
	Primary clarified	62	99
	Activated sludge	37	10
	Secondary clarified	10	<1

[a] Raw count considered 100%.

Bacteroides or a *Sphaerophorus* group. In reality, a number of separate species are represented within each of these groups. On the basis of this grouping, 73% of 105 colonies were *Bacteroides*. The majority of the *Sphaerophorus* group were found as light-blue colonies. A higher isolation rate of this group might result with a more careful study of colonial morphology. Only a few physiological studies were performed among the isolates (Table 1), and these were not primarily intended to identify species.

One discrepancy from the description of the genera does occur (Table 1). Twenty-two strains (21 Group B and 1 Group A) produced nitrite from nitrate, a characteristic listed as negative for both genera as a whole (10; *Bergey's Manual of Determinative Bacteriology*), but omitted in about half of the descriptions of species in each genus. It is possible that these strains represent an undescribed species in human feces. Elucidation of this point must await a more definitive study. In any case, the medium appears to be highly selective for certain members of the closely related genera *Bacteroides* and *Sphaerophorus* that occur in the human intestinal tract and do not require serum or ascitic fluid for growth.

There was some disappointment that the numbers of *Bacteroides* recovered on the selective medium did not reflect the reported (14) abundance of these organisms in the human intestine. Possibly, only a few species of bacteroides are able to grow on this medium, and they occur only in small numbers in the intestine, or the selective agents may exhibit more inhibitory activity to many strains than the preliminary work indicated. It should also be pointed out that these anaerobes are highly susceptible to oxygen and that improvement in handling of samples during collection and incubation to exclude air might result in higher recovery of these organisms. In general, the bacteroides are about 20% of the coliform population in feces (Table 2), and, calculated from Table 3, are about 0.5% of the coliform numbers in raw sewage. This indicates a loss of bacteroides capable of growing on this medium after leaving the body, a loss possibly restricted to certain strains since there seems to be a distinct difference in the distribution (Table 1) of the various physiological subgroups depending on the remoteness of the sample source from the body. Groups B and D were more common in feces, and Groups A, C, and E were most common in the treatment plant samples. The death of certain species more rapidly than others would not be unlikely since these anaerobes are highly sensitive to small amounts of oxygen. Those surviving the treatment plant and beyond may represent the more oxygen-tolerant species or strains. Once sewage enters a treatment plant, the decline in numbers of the bacteroides more or less parallels the decline in coliform numbers (Table 4). This was rather surprising considering the obligatory anaerobic nature of these organisms. Even in the highly aerated activated sludge tanks, significant numbers of bacteroides were recovered. Also to be noted is the recovery of these organisms from the algae pond affluent in Plant A (Table 3). This tertiary treatment step simulates in some respects a stream receiving the discharge from secondary sewage treatment. The water was saturated or supersaturated with oxygen produced by the algae and had a retention time of about 4 hr. Small numbers of bacteriodes were still recovered, indicating that a few of these organisms would likely survive in water under natural conditions even when oxygen is present. There is no doubt that the number of survivors would be low and decline rapidly. Relatively large numbers were obtained from the anaerobic sludge digestion tanks, often at levels exceeding that in raw sewage. Groups A and E were most frequently recovered here, indicating their possible role in anaerobic sludge digestion. Hungate (6) has isolated strains of *Bacteroides* from sludge digestion tanks, but

little other work has been done on this group in sewage.

It is concluded that the selective medium does effectively isolate certain strains of *Bacteroides* and *Sphaerophorus* from feces and from various stages of sewage treatment, including the effluent from aerobic secondary treatment. The disappearance of these organisms during treatment parallels that of the coliforms. The occurrence of these organisms in raw sewage suggests the possibility of their use as indicators of human sewage pollution. However, to be of value for this purpose, considerably more must be known about the natural distribution of the species growing on this selective medium. It is known that bacteroides occur in the intestine of ruminants and pigs, although the few studies with freshly manured soil indicated no bacteroides capable of growing on this medium. Most of our knowledge of the physiology of these genera is based on cultures from human sources. A definitive study of isolates from other natural sources may show considerable differences and would indicate whether certain strains are exclusively human in origin. It is also recognized that the low numbers recovered in sewage and the difficulty of anaerobic work would make this technique doubtful as a routine water analysis, since simplicity is an important feature of routine techniques. However, the results of this study appear sufficiently encouraging to warrant further study of the medium and the bacteroides as an aid in study of the ecology of these anaerobes and possibly to provide an additional measure of human pollution if it is found that some of these strains are unique to human feces.

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