Isolation of Ureolytic *Peptostreptococcus productus* from Feces Using Defined Medium; Failure of Common Urease Tests

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Colony counts of fecal samples from three persons, obtained by using a chemically defined anaerobic roll-tube medium (containing glucose, maltose, glycerol, minerals, hemin, B-vitamins, methionine, volatile fatty acids, sulfide, bicarbonate, agar, carbon dioxide (gas phase), and 1 mM NH_4^+ as main nitrogen source), averaged 60% of the 8.8×10^{10} bacteria per g obtained when 0.2% Trypticase and 0.05% yeast extract were added to the otherwise identical medium. When 0.2% vitamin-free Casitone replaced Trypticase and yeast extract, counts were 94% those of the more complex medium. When urea-nitrogen was added to the defined medium as the main nitrogen source in place of NH_4^+, counts of relatively large colonies averaged 1.0×10^9 per g of feces from five persons—1.1% of counts on the medium containing Trypticase and yeast extract. All of the organisms from the large colonies in the urea roll tubes were morphologically similar, and all six representative strains isolated were identified as urease-forming *Peptostreptococcus productus*, a species not previously known to produce urease. Ureolytic strains of *Selenomonas ruminantium* and *P. productus* were negative for urease activity in three assay media when inocula were from media containing complex nitrogen sources. The study documents that *P. productus* is the most numerous ureolytic species so far found in human feces and suggests that NH_4^+ and more complex organic nitrogen sources strongly repress its production of urease. The study also indicates the efficacy of chemically defined media for direct selective isolation of nutritional groups of bacteria from feces.

Production of NH_4^+ from urea by urease-producing bacteria of the large bowel is a very important aspect of nitrogen metabolism in both the normal and pathological physiology of animals, including humans (17). However, there is a paucity of information on the species of bacteria of functional significance in urease production in the normal bowel. Sabbaj, Sutter, and Finegold (14), in a study of feces of seven patients with Laennec's cirrhosis and acute hepatic coma, isolated and identified ureolytic strains of various facultatively anaerobic bacteria and the anaerobes, *Clostridium perfringens* and *Bacteroides* species. With the possible exception of the latter organism, the species isolated represent less than 0.1% of the normal fecal flora (12).

On the basis of knowledge on the nutritional requirements of rumen anaerobes (2), we recently isolated significant numbers of urease-forming *Selenomonas ruminantium* from rumen by using a chemically defined roll-tube medium containing urea as the main source of nitrogen

(9). This species is well known as a major component of the rumen microbiota (2, 3), but ureolytic strains had not been previously detected.

Because media for rumen bacteria (5, 7, 12) are efficacious for nonselective isolation and enumeration of human fecal bacteria, the present study was initiated to see if the above urea medium would be useful for selective isolation of urease-forming bacteria from human feces. We demonstrate that large numbers of bacteria from human feces can be directly grown in chemically defined roll-tube media, that large numbers of urease-forming *Peptostreptococcus productus*, a species not previously known to produce urease, are present, and that some commonly used urease tests may yield unsatisfactory results with this species and with the ureolytic *S. ruminantium*.

MATERIALS AND METHODS

Anaerobic culture technique. The anaerobic methods used were essentially those of Hungate (8) as

modified (1). Anaerobic conditions were maintained throughout preparation, sterilization, and inoculation of media and dilution of feces.

Roll-tube media. The anaerobic nitrogen-free basal roll-tube medium was that indicated by John et al. (9), except that 0.1% glycerol replaced cellobiose, and only acetate, isobutyrate, *n*-valerate, isovalerate, and DL-2-methylbutyrate were included in the volatile acid mixture. Composition of the basal roll-tube agar medium was as follows: 0.1% each of glucose, glycerol, and maltose; 5.0% mineral 3 (1.8% each of NaCl and KH_2PO_4, 0.04% $MgCl_2 \cdot 6H_2O$ and $CaCl_2$, 0.002% $CoCl_2 \cdot 6H_2O$, and 0.02% $MnCl_2$); 0.0001% hemin, 0.3% volatile fatty acid mixture (36 ml of acetic acid, 1.8 ml of isobutyric acid, and 2.0 ml each of *n*-valeric, DL-2-methylbutyric, and isovaleric acids); 0.5% vitamin solution (20 mg each of thiamine-hydrochloride, calcium-D-pantothenate, nicotinamide, riboflavine, and pyridoxine-hydrochloride; 1 mg of *p*-aminobenzoic acid; 0.25 mg each of biotin and folic acid; and 0.1 mg of vitamin B_{12}; 100 ml of water); 0.1 mM L-methionine, 1.5% Ionagar no. 2, 0.0001% resazurin; 0.4% Na_2CO_3; 0.038% $Na_2S \cdot 9H_2O$; and CO_2 gas phase. Preparation procedures were as described by John et al. (9).

Five different media were made by addition of various components to the basal medium as follows (expressed as final percentage): (1) 0.2 Trypticase (BBL), 0.05 yeast extract (Difco), and 1.0 NH_4Cl solution (100 mM); (2) 0.2 vitamin-free Casitone (Difco) and 1.0 NH_4Cl solution (100 mM); (3) 1.0 NH_4Cl (100 mM); (4) 1.0 urea solution (50 mM, filter-sterilized by passage through a 0.45-μm diameter Millipore filter and equilibrated with CO_2); and (5) no additions. Before gassing with CO_2 and autoclaving, the pH of each medium was adjusted to 6.5 with 10% NaOH. After autoclaving and cooling, the sterile CO_2-equilibrated Na_2CO_3 and urea were anaerobically added to the media contained in round-bottom flasks. The media were then anaerobically tubed in 9.4-ml amounts in sterile 18 × 150 mm rubber-stoppered culture tubes. Just before inoculation, 0.15 ml of sterile Na_2S reducing solution was added to each tube.

Fecal samples and inoculation procedure. The methods used were as described by Eller et al. (5) except that the anaerobic dilution solution, containing minerals, Na_2CO_3, resazurin, Na_2S, and CO_2 gas, as in the basal roll-tube medium, was nitrogen-free. The freshly voided feces of five healthy adults (four males, one female), 24 to 30 years of age were used. No restrictions were placed on diet. Four replicate tubes of each medium were inoculated with 0.5 ml of the dilutions representing 5 × 10^{-9} and 5 × 10^{-10} g (wet weight) of feces. Colonies were counted after 3 and 7 days of incubation at 37 C.

Isolation of ureolytic bacteria. After 3 days of incubation, distinctively large colonies in the urea medium roll tubes were picked with a platinum-iridium needle and stabbed into agar slant medium. The slant medium was the same composition as roll-tube medium containing urea-nitrogen, except that the agar was reduced to 0.7%. These tubes were then incubated until good growth was apparent,

usually overnight; or, as in the case with the small colonies selected, growth was allowed to proceed for 3 days because of poor growth. The slant cultures were then tested for their ureolytic ability by growth in liquid medium with urea as the only nitrogen source. Three liquid media were prepared with the same composition as the roll-tube basal medium but minus the agar and with either 2 mM urea-nitrogen, 2 mM NH_4^+-N, or no added nitrogen, as a control. A 1-ml amount of the control medium was added to the urea slant culture and carefully mixed with the water of syneresis and surface growth. From the resulting suspension, 0.1 ml was inoculated into duplicate tubes of each of the three liquid media. Growth was then followed by measuring the optical density at 600 nm with a Spectronic 20 spectrophotometer (Bausch & Lomb).

Detection of urease activity. Difco Urea Broth (4) and the medium used by Sabbaj et al. (14 and personal communication with Sabbaj) were used to detect urease activity of the fecal isolate and *S. ruminantium* strain D (9), another known ureolytic strain. The former method was slightly modified from that used for facultatively anaerobic enteric bacteria by using N_2 gas phase to allow anaerobic conditions to be maintained during the incubation period which was 72 h rather than 24 to 48 h. Urea broth contains 0.01% yeast extract, 0.91% KH_2PO_4, 0.95% Na_2HPO_4, 2.0% urea, and 0.001% phenol red. It was sterilized by filtration and tubes in 0.5-ml amounts in 13 × 100 mm tubes with an N_2 gas phase. The Sabbaj medium contains 0.1% glucose, 0.5% NaCl, 0.2% yeast extract, 0.2% KH_2PO_4, 2.0% KH_2PO_4, and 2.0% urea. It was also sterilized by filtration and tubed under N_2 in 0.5-ml amounts. These media were heavily inoculated with several loopsful of culture from cultures incubated for 24 h in urea slants or maintenance slants (5) which contained (expressed as final percentages): 0.05 each of glucose, cellobiose, and soluble starch; 0.5 Trypticase; 30.0 rumen fluid; 0.05 cysteine; 0.0001 resazurin; 1.5 agar (Difco); 0.40 Na_2CO_3; minerals, and CO_2 gas phase. The experimental cultures were incubated at 37 C, and daily observations were made to detect changes in indicator color (pH). After 72 h, a qualitative spot test for NH_4^+ production (Nessler reagent) was done. Control reaction mixtures identical with the experimental tubes but lacking urea were included.

Identification and characterization of ureolytic strains. Six strains were identified with procedures of the Anaerobe Laboratory, Virginia Polytechnic Institute (7).

RESULTS

Total colony counts. Data in Table 1 indicate that the mean total count of 8.3 × 10^{10} per g of feces (wet weight) obtained in medium which contained defined ingredients with the exception of vitamin-free Casitone, was only slightly lower, i.e., 94% of the count obtained with medium 1, which was supplemented with Trypticase and yeast extract. The latter me-

TABLE 1. *Colony counts from fecal samples of three humans obtained by using defined roll-tube media with additions of various sources of nitrogen and other growth factors*

Additions to medium[a]	No. of colonies[b]			Bacteria per g[c]
	0–1 mm diam	1–2 mm diam	>2 mm diam	
Trypticase, yeast extract, NH₄Cl	8 (4–10)	17 (11–23)	20 (17–23)	8.8 (8.0–10.0)
Casitone	6 (3–13)	17 (9–31)	18 (13–24)	8.3 (6.4–11.6)
NH₄Cl	7 (4–13)	10 (7–12)	9 (3–14)	5.3 (4.6–5.6)
Urea	18 (15–24)	0	1 (1–1)	3.9 (3.2–5.6)
None	16 (12–19)	0	0	3.2 (2.4–3.8)

[a] See Materials and Methods for the composition of the basal roll-tube agar medium.

[b] The number of colonies represent means for four replicate tubes after 3 days of incubation from each of three samples, each inoculated with 0.5 ml of the 10^9 dilution of feces from a different person. Figures in parentheses indicate ranges.

[c] Figures shown to be multiplied $\times 10^{10}$. Figures in parentheses indicate ranges.

dium is similar in composition to that previously shown to give close-to-maximal counts of fecal bacteria (5). Colony sizes in these two media were similar. When the Casitone was deleted so that the medium was completely defined and with NH_4^+ as the nitrogen source (medium 3), the mean count of 5.3×10^{10} was considerably lower but still about 60% that of the complex medium. Although colony sizes were still adequate for counting or isolation, larger proportions of them were smaller than those in the more complex medium. In studies of 30 colonies from each medium, less diversity in morphological types of cells seemed to be present in medium 3. It contained about 4 different cell types with the medium-width, gram-negative, short rods predominating, whereas in the complex medium, 10 different morphological types were noted with the medium-width gram-negative rods again being the most numerous of the organisms identified. In medium 5 with no nitrogen added, colonies were very small but still present, indicating a small amount of nitrogen contamination. Other experiments suggested that the deletion of methionine did not affect the size of these small colonies. The counts at 3 days were 80 to 95% of those at 7 days for the media with complex

nitrogen sources and 65 to 75% for those with urea or NH_4^+ only added.

Medium 4, containing urea as the main nitrogen source, was of particular interest in that a few large colonies developed which were very easily distinguished by size and density from those developing on medium 5 with no added nitrogen. The data (Table 1) indicate an average of one large colony per tube. However, the roll tubes inoculated with 10-fold greater amounts of feces from these three persons and two others contained an average of 5.5 of these larger colonies (1.1×10^9 per g of feces) and represented 1.8, 0.7, 0.2, 1.0, and 1.9% of the total bacteria cultured in the complex medium 1.

Ureolytic isolates. That these large colonies in medium 4 were urea-utilizing bacteria was confirmed by isolation of strains from 20 large colonies derived from samples from the 5 individuals and demonstration of good growth of all of them in liquid defined medium with either urea or NH_4^+ as the sole source of nitrogen (see Fig. 1 for representative results). Ten strains derived from small colonies exhibited significant growth only on the medium containing NH_4^+.

All the large colonies were of similar size and appearance and contained organisms of similar and typical morphology. They were gram positive, relatively large cocci, 0.8 to 1.2 μm wide by 1.0 to 2.0 μm long (average about 1.0 $\times$ 1.5 μm). The cells were lancet-shaped with pointed ends and occurred with some chains. One strain had
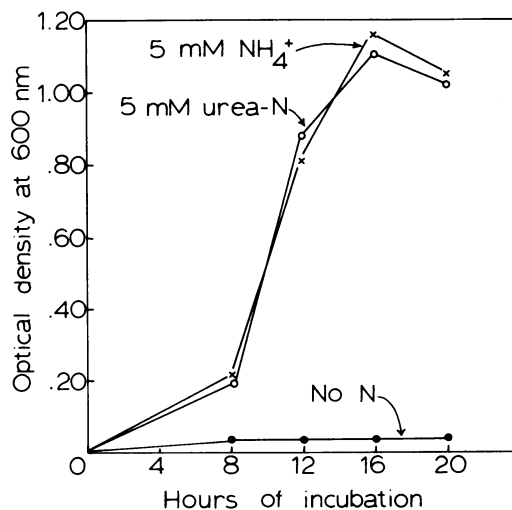

FIG. 1. *Growth of strain 1-1 of Peptostreptococcus productus in chemically defined medium with 5 mM urea-nitrogen* ($\times$), *5 mM NH_4^+-N* (O), *or no nitrogen* (●) *added as sole nitrogen source.*

somewhat smaller cells, averaging about 0.8 µm in width, but otherwise it was identical with the others. Six strains, one from each person plus the small one, were identified as *P. productus* (7). Among other features, they were negative for indole production, nitrate reduction, growth in 6.5% NaCl, NH_3 production from peptone or threonine, catalase production, and lactate fermentation. They fermented many carbohydrates such as arabinose, cellobiose, esculin (hydrolysis usual), lactose, maltose, mannitol, raffinose, sorbitol, sucrose, xylose, and glucose but not dulcitol, or glycogen; starch was not hydrolyzed. Acetate and succinate were the main acid products from glucose fermentation and H_2 was produced. Using media similar to those of Bryant and Robinson (2), we determined that these strains could not use 1.5% Casitone as energy source, B-vitamin(s) were essential, hemin was not required, and a mixture of free L-amino acids (similar to casein in amino acid proportions) served effectively as the nitrogen source in place of NH_4^+ or urea.

Qualitative urease assays. An experiment representative of several experiments (Table 2) showed that, when inoculum was derived from the maintenance slant which contained a large amount of NH_4^+ and organic nitrogen from Trypticase and rumen fluid, the assays for urease with both *P. productus* and *S. ruminantium* were negative or only slightly positive. Results were much more positive when inoculum was derived from the slant containing a small amount of urea as nitrogen source. This suggests that the ammonia or organic nitrogen sources, or both, in the maintenance slants and possibly that in the assay medium repressed formation of urease and that little growth or synthesis of urease by these anaerobic bacteria occurred in the assay media, i.e., the activity shown was largely the result of urease synthesis in cells grown in the defined urea agar slants. It is evident that when inoculum is derived from cultures grown on complex medium, as is usually the case, these assays are unsatisfactory. In support of this, the VPI urease assay which involves complex media (7) was also negative for all six strains of urease-forming *P. productus* which were characterized in detail.

These results (Table 2) show also that the NH_4^+ indicator of activity was more sensitive than the pH indicator. However, more sensitive pH indicator systems are available than that used here (15).

DISCUSSION

Results on enumeration and isolation from all five persons sampled indicate that the anaero-

TABLE 2. *Detection of urease activity in the anaerobic species,* Selenomonas ruminantium *strain D and* Peptostreptococcus productus *strain 1-2 using modifications of two methods in conjunction with two media for inoculum*

Assay medium[a]	Indicator[b]	Urea slant		Maintenance slant	
		Strain D	Strain 1-2	Strain D	Strain 1-2
Difco Urea Broth	pH	+[c]	+++	0	0
		0	+	0	0
	NH_4^+	+++	+++	±	±
		+++	+++	±	±
Sabbaj et al.	NH_4^+	++	++	0	0
		+++	+++	0	0

[a] See Materials and Methods for composition of media. Medium was heavily inoculated by scraping the surface of the urea or maintenance slant. Incubation was for 72 h at 37 C.

[b] Change in indicator color (pH) was observed, and then a qualitative spot test for NH_4^+ (Nessler reagent) was done.

[c] The scale used was 0 to +++ as compared to identical assay medium culture control without urea. Duplicate tests within one experiment are indicated.

bic species *P. productus* may be the most important functional ureolytic bacterium thus far isolated from human feces. Good documentation on the numbers of ureolytic bacteria found in the lower gut is difficult to find. Sabbaj et al. (14) found an average number of about 10^8 ureolytic bacteria per g (dry weight) of feces (about 10^7 based on wet weight) in patients with cirrhosis and acute hepatic coma. Although 10^9 cells of ureolytic *P. productus* were shown in the present study, a valid comparison cannot be made because of the difference in health of the persons studied. Among the 11 ureolytic organisms (mostly facultative anaerobes) isolated by Sabbaj et al., none, with the possible exception of *Bacteroides* species, is usually found at a level of 10^9 per g of wet feces as the *P. productus* strains were. Phear and Ruebner (13) indicated that no urease activity was observed in the strains of *Bacteroides* with which they worked. Nutritional studies have indicated that *B. fragilis* strains would grow very well in our medium with NH_4^+ (16). Since none of them was found in the urea medium of the present study, it is evident that ureolytic biotypes of *B. fragilis*, if they exist at all, are present in very low numbers. Gibbons and Doetsch (6) isolated ureolytic strains of *Bifidobacterium* from the rumen. Matteuzzi and Crociani (11) indicated that, among 17 species of this genus, only

strains of *Bifidobacterium suis* from swine produce urease; however, we are unable to evaluate this study since growth media were not indicated and urease might have been repressed in some strains.

Although ureolytic species other than *P. productus* were not detected in the high dilutions of fecal material cultured in the present study, we know that other species are present. In work in progress (Mary Ann Wozny and M. P. Bryant), we have developed a new medium for rapid assay of urease in anaerobic bacteria which may have complex nutrient requirements. Using nonselective rumen fluid-based roll-tube medium (3, 7), we have demonstrated that urease-forming organisms accounted for 14% (range of 7 to 22%) of 382 strains randomly isolated from four fecal samples. The 54 urease-forming isolates have not yet been identified, but most are morphologically similar to *P. productus*, four are gram-positive, nonmotile, slender, anaerobic rods, and two are gram-negative, slender, motile, anaerobic rods. Whether these nonselectively isolated organisms require nitrogen sources or other factors not included in the defined medium used in the present study is not yet known. Unpublished data (Janice Herbeck and M. P. Bryant, 1974) show that strains of *Ruminococcus bromii*, a major bacterium of human feces, require pantethine and tetrahydrofolate for growth and therefore will not grow in the present defined medium. This species also needs a branched-chain acid such as isobutyrate. It is also possible that some important species of the proximal large bowel are greatly reduced in numbers by dehydration that occurs during passage of the fecal mass through the large bowel.

Preliminary unpublished results of Betty Cato (VPI Anaerobe Laboratory) suggest that most strains of *P. productus* isolated with nonselective media produce urease. All four strains of *P. productus* type I and three strains of *P. productus* type II (12) were positive for urease when assayed with the defined media of the present study. These two types of *P. productus* represented 4.23×10^{10} bacteria per g (dry weight) and ranked second among species in frequency of isolation from feces in a recent study (12).

A satisfactory routine method for the rapid detection of urease activity in anaerobic bacteria has not yet been developed. Recent results with *S. ruminantium* (9), results of this study (Table 2), and studies in progress in this laboratory (M. A. Wozny and M. P. Bryant) all indicate that urease in both *P. productus* and *S.*

ruminantium is strongly repressed by NH_4^+ and complex organic nitrogen sources. This phenomenon is well known among bacteria capable of growth under aerobic conditions (10), and good routine assay procedures have been developed for aerobic bacteria in which urease is often repressed, e.g., see the study of Stewart on pseudomonads (15). However, most qualitative urease assays presently used to study anaerobes are not satisfactory.

The results showing (i) the chemically defined medium plus Casitone gave very high colony counts similar to those of the complex medium, and (ii) those on the selective isolation of ureolytic bacteria indicate that similar methods might be satisfactory for the direct numerical estimation of various other nutritional features of the bacteria of the colon. It was previously suspected that, although many of the bacteria of feces or of the large bowel might have simple nutritional features (5), many of the cells would be in an injured state and show low viability, i.e., low numbers of colonies would be grown on primary culture in agar medium, unless complex medium were used. This study shows that large numbers of cells in feces are viable even in very simple defined medium, for example, one with NH_4^+ as nitrogen source. It would be of considerable interest to compare viability of bacteria in feces with those in the proximal large bowel, the area in which most of them are presumed to grow, in complex versus relatively simple chemically defined culture medium. It is possible that many injured bacterial cells of the proximal large bowel die and lyse during passage through the bowel.

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