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Increase in Colonic Methanogens and Total Anaerobes in Aging Rats

ANNE E. MACZULAK,† MEYER J. WOLIN, AND TERRY L. MILLER*

Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, New York 12201–0509

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Methanogens are present in the colons of our local Wistar rat colony. We studied the changes in concentrations of their fecal methanogenic and nonmethanogenic bacteria with age as a model of the development of these communities in humans. We found that the predominant methanogen in the rats is a *Methanobrevibacter* species. The log of the concentration of total anaerobes increased from 9.8/g (dry weight) at 3.0 weeks of age (shortly after weaning) to 10.7/g (dry weight) at 96 weeks (shortly before the end of the life span). In contrast, the log concentration of methanogens increased from 5.5 to 9/g (dry weight) during the same time period. Therefore, methanogens increased as a percentage of the total anaerobes from 0.005% at 3.0 weeks to 2.0% at 96 weeks. About 12 doublings of the methanogenic population and 3.3 doublings of the nonmethanogenic population took place from weaning until death. The slow increase in the ratio of methanogens to total anaerobes with age followed the same pattern in cecal contents as found in feces. There were no relationships between animal weights or fecal outputs and the increase in total anaerobe and methanogen concentrations in feces. A possible explanation for the slow increase in the *Methanobrevibacter* species in Wistar rats with age is a gradual shifting of the use of electrons from the reduction of CO_2 to acetate by acetogens to the reduction of CO_2 to CH_4. The results provide the first evidence for an age-related change in the nonmethanogenic bacteria of the colon and supporting microbiological evidence for physiological studies that have shown age-related increases in colonic methane production in humans.

Methanogens are the only known archaebacteria that inhabit the intestinal tract. They are present in the ruminant forestomachs and in the colons of many monogastric herbivores and omnivores (15). Methanogens produce most of the CH_4 formed in these systems by using H_2 to reduce CO_2 to CH_4. There is little information about how methanogens colonize these ecosystems, i.e., how inoculation occurs and the factors that control the increase in the concentrations of the methanogens in the ecosystems. Their concentrations depend on the production of H_2 by the nonmethanogenic flora and fauna. The nonmethanogenic microorganisms also form acetate, propionate, butyrate, and CO_2, the other major products of intestinal tract fermentations (5, 25).

Although colonization by methanogens of the rumen appears to be ubiquitous, physiological and microbiological studies show that colonization of the human colon by methanogens is not the same in all humans (1, 4, 23). Methane is produced in large amounts in some adults but not in others. Although the amount of CH_4 produced varies widely among individuals, it is relatively constant during the life of an adult (1). The concentrations of the methanogen apparently responsible for using colonic H_2 to reduce CO_2 to CH_4, *Methanobrevibacter smithii*, varies between fewer than 10 to 10^{10}/g (dry weight) of feces between individuals but are relatively constant in an individual (13, 23). The concentrations of *M. smithii* in feces of about one-third of adults on Western diets are between ca. 10^8 and 10^{10}/g (dry weight), which we have estimated to correspond to the production of 0.03 to 3 liters of CH_4 per day (15, 23). The latter amounts are sufficient to lead to the excretion of greater than ca. 1 ppm (μl/liter) of CH_4 in breath (1). About 20% of the CH_4 produced in the colon is absorbed into the blood, transported to the lungs, and excreted in breath (1). CH_4 concentrations in breath higher than ambient were not detected in children of less than 2 years of age but were found in children of between 2 and 10 years of age (1, 19).

Because of the unique characteristics of methanogens, selective procedures can be used to study their colonization of intestinal ecosystems. Their resistance to antibiotics that inhibit eubacteria facilitates isolation, characterization, and enumeration of methanogens. Fluorescence of a unique electron transport coenzyme, factor 420, allows direct microscopic visualization of methanogens in the presence of large concentrations of eubacteria. We applied these methods in the present study to investigate the colonization of the intestines of Wistar rats by methanogens. Lajoie et al. (9) showed that fecal suspensions of adult Wistar rats from the Wadsworth colony harbored a methanogenic flora, whereas those from a colony of the DA strain of rats did not harbor fecal methanogens. Comparative studies of the two colonies may provide clues to the reasons for differences in the colonization of the human colon. In this report, we describe the colonization of Wistar rats by methanogens and nonmethanogenic anaerobes. We show that the methanogen responsible for using H_2 to reduce CO_2 to CH_4 in the Wistar strain is a *Methanobrevibacter* species similar but not identical to *M. smithii*. We also describe how its concentration and the concentration of total viable anaerobic bacteria change in feces of the rats at between 3 and 96 weeks of age.

MATERIALS AND METHODS

Animals. All rats used were from a Wistar rat colony maintained by the central animal facility of the Wadsworth Center. The colony was established in 1959 from animals obtained from the Walter Reed Army Hospital and has been maintained by random breeding. Ten randomly selected rats, five males (90 to 120 g) and five females (90 to 100 g), 3 weeks of age, were used to examine the concentrations of metha-

* Corresponding author.
† Present address: Gillette Research Institute, Gaithersburg, MD 20879.

nogens and total anaerobes in feces of aging rats. Total concentrations of methanogens and total anaerobes in ceca were studied with male rats at 3, 6, 9, and 14 weeks of age (four rats of each age). The location of total anaerobes and methanogenic bacteria in different colonic sites was determined in a 26-week-old male. All rats were housed separately by sex (two to three rats per cage) in cages lined with wood shavings. Fecal samples were collected from individual rats after they were placed in a metabolism cage. Fecal samples from groups of rats were collected after they were placed in cages fitted with raised wire floors. All rats were fed commercial laboratory chow (Ralston Purina Co., St. Louis, Mo.), provided with water ad libitum, and adapted to a 12-h light/dark cycle and a temperature of 20 to 22°C. Rat fecal and colonic experimental protocols were approved by the Wadsworth Animal Welfare Committee in June 1983, and protocols were reviewed and approved yearly thereafter.

Fecal and colonic samples. The time elapse from defecation to laboratory processing of the fecal pellets was ca. 1 h for individual rats and ca. 20 min for pooled samples. For the determination of bacterial concentrations, 2 g of fecal pellets was collected from each rat weekly from 3 to 15 weeks of age and then at 18, 22, 26, 34, 47, 58, and 62 weeks. One male rat died after the 47-week measurements. Analyses at 70 and 72 weeks were done with pooled fecal pellets collected from male and female rats, respectively. At 80 and 96 weeks, pooled fecal pellets from males were analyzed. By the age of 80 weeks, only three female rats were alive. Because the data collected before this age indicated that there were no sexual differences (see Results), the remaining females were removed from the study.

Suspensions (10%, wt/vol) were prepared in anaerobic dilution solution (ADS [2]) reduced with 0.05% (final concentration) each of dithiothreitol and sodium sulfide. The initial suspension was mixed by the stomacher method (13) and used for enumeration. Fecal dry weights were determined as described previously (13). Body weights for each rat were recorded weekly through 15 weeks and monthly thereafter until 62 weeks of age. Total feces production over 24 h was also recorded for each rat until 62 weeks of age.

For the determination of concentrations of methanogens and total anaerobes in ceca, rats were sacrificed by CO_2 asphyxiation. Hindgut sections were occluded at the ileocecal juncture and at the distal end of the rectum with hemostats. After the hindgut was removed from the rat, it was transported on ice to an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) which contained a gas mixture of N_2-H_2-CO_2 (85:10:5). Total digesta from the cecum, colon, or rectum was transferred to a sterile bottle and mixed. A 0.5- to 1.0-g subsample was diluted 1:10 in ADS and used in enumeration methods.

For the determination of the distribution of methanogens in the colon, a rat was sacrificed by CO_2 asphyxiation. Hindgut sections, including the cecum, postcecal colon, and the distal colon-rectum region, were separated using hemostats, removed from the rat, and held on ice until transported to the anaerobic chamber. Luminal contents from each hindgut region were removed, diluted in sterile ADS, and used in the enumeration methods. Tissue pieces (1.0 cm^2) were cut from the cecum with a sterile scalpel and template. Each tissue sample was washed sequentially six times in 9.0 ml of sterile ADS by shaking manually for 30 s and decanting the supernatant. After the sixth washing, the tissue sample was transferred with sterile forceps to a 100-ml serum bottle containing 9.0 ml of ADS and was homogenized (Ultra-Turrax tissue homogenizer, model SDT; Tekmar Co., Cincinnati, Ohio) for 2 min while gassing with CO_2. The supernatants and homogenate were used as inocula for enumeration procedures.

Enumeration of bacteria. The media used for the enumeration of total viable anaerobes and methanogens were the complex ruminal fluid-based agar media (BRN media) previously described for human fecal enumerations (12) except that 0.05% (final concentration) each of dithiothreitol and sodium sulfide were used as the reducing agents. Serial 10-fold dilutions were prepared in ADS from the suspensions of fecal or gut contents and used as inocula for the roll tube media. For total anaerobes, 0.5 ml of the appropriate dilutions was inoculated into duplicate roll rubes containing the BRN medium and 101.3 kPa of CO_2. Methanogens were enumerated similarly except that the BRN medium contained cephalothin (6.7 μg/ml) and clindamycin (1.7 μg/ml) and a gas phase of 101.3 kPa of 80% H_2–20% CO_2. The filter-sterilized antibiotics were added aseptically to the molten agar medium before inoculation. Roll tubes were incubated statically at 37°C for 14 days. An incubation period of 14 days was adequate for the growth of colonies to a sufficient size for the determination of colony counts. The number of colonies did not increase with incubation beyond 14 days. CH_4 production in the antibiotic-containing roll tubes was determined by gas chromatography (3).

Isolation and characterization of methanogens. Colonies were picked from CH_4-positive antibiotic roll tube platings of two males (9 and 14 weeks of age) and two females (10 and 14 weeks of age). They were transferred to liquid BRN medium with antibiotics (12) and 101.3 kPa of 80% H_2–20% CO_2.

Isolates were tested for the ability to use formate, acetate, methanol (alone or with H_2), and trimethylamine as methanogenic substrates (16) in phosphate-buffered ruminal fluid medium (14). They were tested for growth in media of various complexities with 80% H_2–20% CO_2 as the substrate (16). Bile sensitivity was tested in BRN liquid medium with 2% oxgall and 0.1% sodium deoxycholate with 202.6 kPa of 80% H_2–20% CO_2 (16). The pH optima were determined in phosphate-buffered complex ruminal fluid media in which the total phosphate was 0.05 M and the total $NaHCO_3$ and CO_2 was 300 μmol at any given pH (14). The gas phase also contained H_2 and N_2. The temperature range of growth was determined in BRN medium with 202.6 kPa of 80% H_2–20% CO_2. Anaerobic biphasic culture techniques (17) were used to obtain cells for DNA isolation (14). The moles percent G+C was determined by thermal denaturation (14). Values of the isolates are the average of at least three determinations, with *Escherichia coli* ATCC 11775 included as a reference in each determination.

RESULTS

Fecal methanogen isolates. The logarithms of the methanogen concentrations for the two males and two females were 8.18, 7.88, 6.94, and 8.04/g (dry weight), respectively. Methanogens were isolated from the fecal platings of each animal. Each isolate produced CH_4, had a uniform cell morphology, and showed factor 420 fluorescence of all cells in a field when viewed under epifluorescence microscopy. The isolates did not grow in complex media in the presence of carbohydrate and the absence of a substrate for methanogenesis.

Isolates from all animals were coccobacilli and grew and produced CH_4 from H_2 and CO_2. Three of the four isolates grew poorly with formate as a methanogenic substrate; the

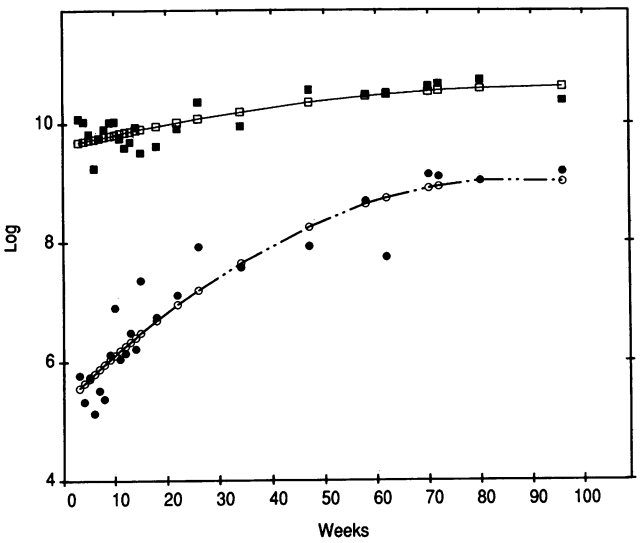

FIG. 1. Total anaerobe and methanogen concentrations (log per gram [dry weight]) and rat age (weeks). Means of total anaerobes: ■, observed values; □, predicted points of the second-degree polynomial equation $-1.01 \times 10^{-4} x^2 + 0.02x + 9.63$ ($F = 20$, significance level = 0.001; $R^2 = 0.66$). Methanogens: ●, observed values; ○, predicted points of the second-degree polynomial equation $-4.96 \times 10^{-4} x^2 + 0.09x + 5.30$ ($F = 89$, significance level = 0.0001; $R^2 = 0.89$.

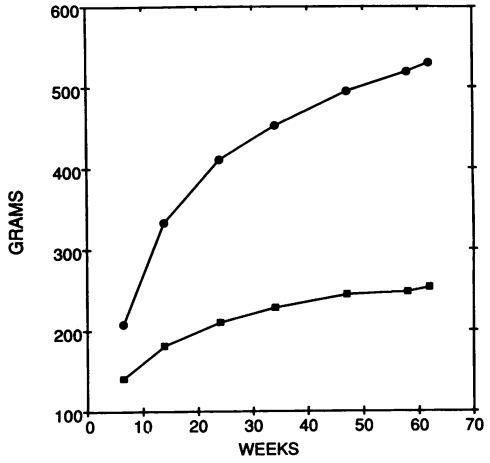

FIG. 2. Rat weights (grams) and age (weeks). Symbols: ●, males; ■, females.

other isolate did not grow with formate. None used acetate, methanol (with or without H_2 as an electron donor), or trimethylamine as a methanogenic substrate. The isolates did not require 2-mercaptoethanesulfonic acid or branched-chain volatile fatty acids for growth. Growth was inhibited by bile. All isolates had a temperature optimum at 35 to 41°C, grew poorly at 32°C, and did not grow at 44°C. The optimal pH for growth was 7.0. The moles percent G+C of the four isolates ranged from 31.7 to 33.9 (mean, 32.6 mol% G+C).

Methanogens, total anaerobes, and age. The changes in total anaerobe and methanogen concentrations in feces as a function of age are shown in Fig. 1. The datum points are averages of the results for males and females for all periods except for 80 and 96 weeks, when only male rats were available. The curves were drawn by fitting the data to a second-degree polynomial. Curves fitted to separate datum points for males and females, respectively, coincided with the curves shown in Fig. 1. Therefore, no differences in changes in concentrations between males and females were detected. The log of the concentration of total anaerobes increased from 9.7/g (dry weight) at 3 weeks of age (shortly after weaning) to 10.6/g (dry weight) at 96 weeks (shortly before the end of the life span). In contrast to this increase of approximately 1 log, the concentration of methanogens in feces increased 3.5 logs during the same period, from 5.5 to 9.0/g (dry weight). Therefore, methanogens increased as a percentage of the total anaerobes from 0.005% at 3 weeks to 2.0% at 96 weeks. Factor 420 fluorescent coccobacilli were readily observed by epifluorescence microscopy in 10% fecal suspensions of all rats after concentrations of methanogens were ca. 10^6/g (dry weight) of feces.

Weight gains and fecal output. The weights of the animals as a function of age are shown in Fig. 2. The rate of increase in weight with age was faster than the rate of increase of the log of total anaerobe and methanogen concentrations in feces. Since daily fecal output remained relatively constant for both male and female rats from 4 to 62 weeks of age, there was no relationship between fecal output and the change of methanogen or total anaerobe concentrations with age. The mean daily fecal dry weight outputs for males and females were 5.2 ± 1.3 and 3.8 ± 1.2 g, respectively.

Age and cecal contents. Comparisons of the concentration relationships in cecal contents with age (Table 1) showed that the slow increase in the ratio of methanogens to total anaerobes followed the same pattern in intestinal contents as found in feces. The ratio of total anaerobes to methanogens was ca. 10^5:1 at 3 weeks and decreased to ca. 10^3:1 at 14 weeks.

Distribution in the colon. The percent distribution of total anaerobes in the colon was 70, 11, and 19 for the cecum, postcecal colon, and distal colon-rectum region, respectively (Table 2). For the same regions, the methanogen percent distribution was 45, 21, and 34, respectively. Although the percent distributions appear to be different, the measurement was only on one sample from one animal. Additional analyses with more animals would be necessary in order to apply appropriate statistical tests to evaluate the significance of the differences. The bacteria in the first wash of the cecal wall tissue were not included in the analysis of tissue-associated bacteria because they were assumed to be mainly of luminal origin. The second wash contained essentially all of the bacteria that could be considered attached but removable by washing. Very few bacteria were found in the subsequent washes as compared with the amounts in the second wash and the amounts that were recovered from the tissue homogenate. The ratios of cecal lumen to wall (second wash plus tissue homogenate) total anaerobes and methanogens were 4,000:1 and 2,000:1, respectively.

TABLE 1. Total anaerobes and methanogens in colon contents[a]

Age (wk)	Dry wt (g)	Total colonic bacteria	
		Anaerobes	Methanogens
3	0.55	4.3×10^{10}	2.4×10^5
6	1.29	8.0×10^{10}	2.0×10^7
9	2.19	4.4×10^{10}	8.3×10^8
14	2.99	9.7×10^{10}	1.1×10^8

[a] Colon contents were from pooled samples from four male rats at each age.

TABLE 2. Anaerobes and methanogens in different colonic locations of a 26-week-old male

Fraction	Total bacteria	
	Anaerobes	Methanogens
Lumen		
Cecum	7.57×10^{10} (70)[a]	2.12×10^{7} (45)[b]
Postcecal colon	1.19×10^{10} (11)	1.00×10^{7} (21)
Colon-rectum	2.11×10^{10} (19)	1.65×10^{7} (34)
Sum	1.09×10^{11}	4.77×10^{7}
Cecal tissue		
Wash 2	1.40×10^{7}	1.04×10^{4}
Washed tissue	4.10×10^{6}	7.00×10^{2}
Sum	1.81×10^{7}	1.11×10^{4}

[a] Percentage of the sum of anaerobes in the lumen of the cecum, postcecal colon, and distal colon-rectum.
[b] Percentage of the sum of methanogens in the lumen of the cecum, postcecal colon, and distal colon-rectum.

DISCUSSION

Methanogens are present in the colons of rats of some, but not all, colonies. Levitt et al. (10) showed that H_2 was rapidly catabolized in the ceca of Sprague-Dawley rats, but CH_4 was not produced. However, Rodkey et al. (21) reported CH_4 production rates of as high as 29 ml/day with Sprague-Dawley rats. DA rats in our local colony do not have methanogens. Their colonic microflora uses H_2 to reduce CO_2 to acetate (9) by the following equation: $2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O$. Acetate synthesis from CO_2 and H_2 by cecal contents from a colony of Wistar rats was demonstrated and no CH_4 was formed (20). However, rats from our local Wistar colony produce both CH_4 and acetate from CO_2 and H_2 (9).

Our results show that the predominant methanogen in our local Wistar colony is a *Methanobrevibacter* species. Its morphology, physiology, and moles percent G+C of its DNA are compatible with those of *Methanobrevibacter* species (15, 17). Its only important substrates for CH_4 production are H_2 and CO_2. An isolate from a H_2-CO_2 enrichment of feces of rats from the same colony had similar characteristics (15, 17) and had a pseudomurein cell envelope (8). However, DNA hybridization studies showed that it did not belong to any of the existing *Methanobrevibacter* species (15). *M. smithii* is the major H_2-CO_2-using methanogen present in the human colon (12, 23). *Methanobrevibacter* species are also found in feces of other animals and the bovine rumen (15, 17).

A possible explanation for the slow increase in the *Methanobrevibacter* species in Wistar rats with age is competition for electrons used by bacteria that reduce CO_2 to acetate (acetogens). The production of H_2 from these electrons would provide the substrate that limits the growth of methanogens in the ecosystem. Electrons normally used by *Acetobacterium woodii* to reduce CO_2 to acetate were preferentially used for CH_4 production when H_2-using methanogens were cocultured with *A. woodii* with fructose as the substrate (24). Preliminary studies indicate that the reduction of CO_2 to acetate is very active when fecal suspensions of young Wistar rats (11 weeks) are incubated with H_2 and CO_2 (unpublished results). Little CH_4 was produced by the low concentrations of methanogens (10^7/g [dry weight]) in the fecal suspension. This contrasts with the approximately equal formation of acetate and CH_4 with similar incubations with older rats with 10^9 methanogens per g (dry weight) (9).

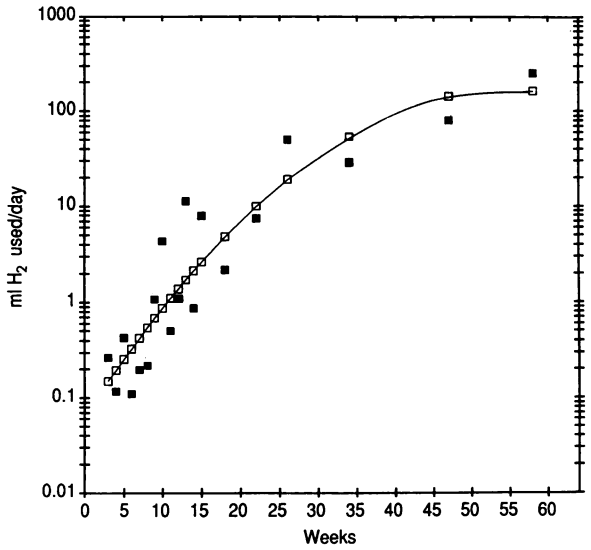

FIG. 3. Calculated amounts of H_2 used per day for growth of methanogens and rat age (weeks). Symbols: ■, calculated values; □, predicted points derived from a second-degree polynomial equation for a plot of the logarithms of the calculated amounts of H_2 versus weeks. The polynomial equation was $-1.14 \times 10^{-3} x^2 + 0.12x - 1.19$ ($F = 44$, significance level = 0.0001; $R^2 = 0.85$.

Acetogens may be able to attain high concentrations in the colon early in the life of the animal because most acetogens are not restricted to H_2 and CO_2 as growth substrates. Many are capable of growth on carbohydrates as well as H_2 and CO_2 (11). As the rats age, the ability of methanogens to compete with acetogens for electrons may increase. More CO_2 would be reduced to CH_4 and the concentrations of methanogens would increase. Additional studies to examine the changes in the relationship between the production of acetate and CH_4 from CO_2 during the life span of the rats are necessary to test the validity of this explanation.

The amount of H_2 necessary for the production of the daily output of methanogens in feces can be estimated if it is assumed that CH_4 production and H_2 utilization are a function of biomass produced, 4 g of biomass is produced per mol of CH_4 formed (4 mol of H_2 used) (15), and the weight and dry weight of methanogen cells are equivalent to the weight of *E. coli* (22). By using the values for methanogens per gram (dry weight) shown in Fig. 1 and measured values of daily fecal dry weight output, the calculated H_2 utilization is shown as a function of the age of the rats in Fig. 3. The curve was drawn by fitting the data to a second-degree polynomial. The amount of H_2 used at 58 weeks was 162 ml/day, based on the value predicted by the polynomial. This is equivalent to the production of 41 ml of CH_4 per day and would be formed by producing 1.5×10^9 methanogens per day. Similar calculations indicate that the highest amount of 29 ml of CH_4 produced per day found for Sprague-Dawley rats (21) would correspond to the production of 10^9 methanogens per day.

The slow increase in methanogen concentrations with age probably is unrelated to rates of cell division. A comparison of the rates of increase of the methanogen population with estimates of growth rates of the cells of the population shows that cell doubling times must be much shorter than population doubling times. The rates of increase in populations at a particular age can be calculated from the slope of the curve at that age (Fig. 1). At 5 weeks of age, the doubling time for

the methanogen population was 3.7 weeks. The doubling times increased with age.

The minimal dilution rate is equal to the minimal growth rate (or maximal doubling time) required for maintaining any species in the colon without washout. The minimal dilution rate can be estimated by assuming that all microbial growth is in the cecum and the rate of liquid entering and leaving the cecum is equal to daily water intake. Cecal volumes of about 10 ml were found for Sprague-Dawley rats fed a high-fiber diet (6). Water intake for adult rats is 24 to 35 ml/day (7). The minimal dilution (growth) rate is 24 ml/day divided by 10 ml, or 2.4 day^{-1}. The corresponding maximal doubling time is 0.33 days, or 8 h. It is unlikely that changes in specific attachment to the colonic wall significantly lengthen the cell doubling required for maintaining a species in the ecosystem. Total numbers of anaerobes and methanogens attached to the cecal wall are several thousand-fold less than the numbers in the lumen (Table 2 and reference 18). The minimal doubling time for the methanogen population was calculated from the slope of the curve of Fig. 1 at 5 weeks. The population doubling time was 3.7 weeks. This is 78 times longer than the estimated maximal cell doubling time of 8 h. This is consistent with the notion that a slow increase in the availability of H_2 for total growth as the rats aged caused the increase in methanogen concentrations and that the cell growth rate was always higher than the dilution rate of the system.

Present evidence suggests that development of methanogen populations in the human colon is also a slow process. Positive breath CH_4 (higher than ambient CH_4 concentrations of ca. 1 ppm) was detected in children between the ages of 2 and 10 years but not in younger children (1, 19). We estimated that breath CH_4 becomes greater than 1 ppm when the methanogen concentration is equal to or greater than 10^8/g (dry weight) of feces (15). If inoculation at birth is assumed, considerable time is required for producing the concentrations of methanogens in the colon required for the production of detectable breath CH_4. Since fecal suspensions of nonmethanogenic or moderately methanogenic humans use H_2 to reduce CO_2 to acetate (9), the suggested explanations for the development of the rat methanogenic population also apply to humans.

Colonization of the rat colon by the nonmethanogenic flora is rapid (18). No appreciable differences in the concentrations of the major populations were found beyond 4 weeks of age (postweaning). Because of the large number of analyses in this study, we found that the total anaerobe concentrations increased by approximately 1 log (about 3.3 doublings) between the period immediately after weaning and very old age (96 weeks). A minimum population doubling time of 16 weeks was calculated from the data shown in Fig. 1. This appears to be the first evidence for an age-related change in the nonmethanogenic bacteria of the colon. A 3.3-fold increase in concentration could reflect a significant increase in the magnitude of the colonic fermentation or significantly greater efficiency of biomass production from the fermentation. However, we cannot be certain that the total biomass of the nonmethanogenic population increases with age. Population changes could influence the plating efficiency of the enumeration procedure. A shift to larger numbers of smaller colony-forming units would also lead to increased total viable anaerobe counts without influencing biomass concentrations. Additional investigations are necessary to determine the nature of the change in the microbial community, whether it indicates a significant change in the fermentation process, and whether there is any relationship to the increase in concentrations of methanogens.

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