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Use of Combusted Natural Gas to Cultivate the Anaerobic Bacterial Flora from the Cecum Contents of Mice

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The use of combusted natural gas as atmosphere for cultivation of the anaerobic bacterial flora from the cecum of mice is described. The combusted gas (inert gas) continuously flushes a glove box in which it is possible to cultivate a number of strict anaerobes from the cecum of mice.

To isolate the anaerobic microflora from the cecum of mice, strict anaerobic conditions are required (1). Recently it became possible to cultivate the tapered rods from the cecum with improved anaerobic techniques (2, 6, 7).

The methods used to obtain anaerobic conditions in the working space can be separated into two groups: (i) a system, whereby an anaerobic atmosphere is continuously maintained (Aranki et al. 1). The atmosphere is carefully kept free of O_2 by means of a catalyst. (ii) The second system is one in which anaerobic conditions in the working space are attained during a limited time (2, 6, 7) by purging the working space with O_2-free gas obtained from a separate gas supply.

It is possible to combine the advantages of both systems by continuously purging the working space with O_2-free gas. In this case the costs of the gas can be ignored. In our study we use a glove box which is continuously purged with O_2-free gas from a so-called inert gas generator.

MATERIAL AND METHODS

Inert gas generator. In the process industry a gas mixture of 12% CO_2 and 88% N_2 is called inert gas. This inert gas is produced by combustion of a gaseous or liquid fuel with air in an inert gas generator, an apparatus with many applications in the industry. For the production of inert gas, a gaseous fuel is preferred, especially for small-capacity equipment, to avoid atomization and gasification problems. We used natural gas as a fuel, which is like all other fuels composed of the elements hydrogen and carbon. When fuel is combusted completely, the C and H components are transformed to CO_2 and H_2O, respectively. The N_2 of the combustion air does not take part in the reaction, except for the formation of a small amount of NO and NO_2. Consequently, the combustion products are composed principally of N_2, CO_2, and H_2O. In the case of an inert gas generator, the gas-to-air ratio is regulated so that the combustion is almost complete. There is no excess of air; on the contrary, there is a very small excess of fuel, causing the formation of approximately 0.3% CO and H_2 in the inert gas. With the help of this CO and H_2, traces of O_2 in the inert gas can be eliminated, using a copper catalyst.

Figure 1 shows the flow scheme of the inert gas generator. A small compressor (Fig. 1, no. 10) sucks the fuel-air mixture from the T-tube (no. 7). In this T-tube, air and fuel are mixed. The fuel is supplied via a stopcock (no. 1), a filter (no. 2), and a magnetic valve (no. 3) at a pressure of 250 mm water gauge (WG). By means of the pressure regulator (no. 4) the pressure is reduced to 50 mm WG. A second pressure regulator (no. 5) is called a "zero pressure gas governor." This regulator reduces the pressure to the same value as the pressure of the combustion air, entering the orifice (no. 8) via an air filter (no. 9). This system makes the combustion process independent of changes in the atmospheric pressure. With the needle valve (no. 6) situated in the fuel line, the amount of fuel entering the T-tube and the fuel-to-air ratio can be adjusted.

The fuel-air mixture is fed to the burner (no. 11). This burner is placed on the combustion chamber (no. 12) and can be removed for ignition by hand. The combustion chamber as well as the surrounding cooling jacket (no. 13) is made of glass, making the flame visible. After combustion, the gases are cooled in the cooling tube (no. 14) which is made of copper and wound in a helical coil. When the combustion products are cooled, the condensation of H_2O starts at about 60 C. The gases are cooled to 20 C and are thus saturated at this temperature (dew point) when leaving the condensate separator (no. 15), which is placed under the cooling tube. The condensate is removed via a watertrap (no. 16). The dew point can be regulated between certain limits by regulating the temperature of the cooling water in the cooling jacket. This occurs by means of a temperature-regulating

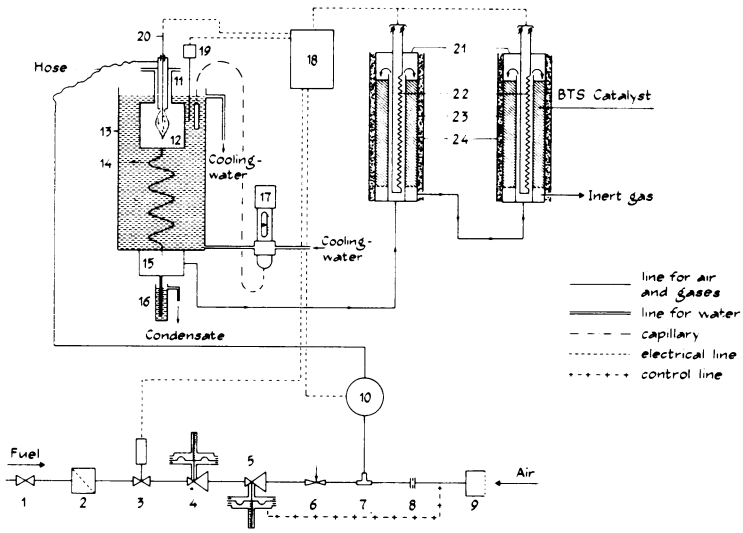

FIG. 1. *Flow scheme of the inert gas generator. Parts: 1, stopcock; 2, filter; 3, magnetic valve; 4, pressure regulator; 5, second pressure regulator; 6, needle valve; 7, T-tube; 8, orifice; 9, air filter; 10, compressor; 11, burner; 12, combustion chamber; 13, cooling jacket; 14, cooling tube; 15, condensate separator; 16, watertrap; 17, regulating valve; 18, control box; 19, thermostat; 20, ionization electrode; 21, catalyst columns; 22, electrical elements; 23, glass-wool blankets; 24, sheet cylinders.*

valve (no. 17). The composition of the inert gas leaving the condensate separator, based on natural gas as a fuel, is as follows: CO_2, 12%; H_2O, dew point ± 20 C; O_2, 30 μliters/liter; CO + H_2, 0.3%; NO + NO_2, trace; N_2, the remainder.

The composition of inert gas has been determined by analysis with regard to the oxygen and by calculation with regard to the remaining gases. The consumption of natural gas is ⅛ m^3 per h, producing 1 m^3 of inert gas per h. The consumption of cooling water and electricity is 720 liters/24 h and 8 KWh/24 h, respectively. The generator is protected against flame failure and failure of the supply of gas, water, and electricity. In case of failure, the generator switches off automatically by means of a control box (no. 18) connected to a thermostat (no. 19) and an ionization electrode (no. 20). The generator is not commercially available. Those who are interested, can obtain from us a comprehensive list of technical details and work-drawings.

Catalyst. It is necessary to remove the traces of O_2 from the inert gas. This is done by means of a B.T.S. copper catalyst (Color Chemie, Arnhem, The Netherlands). This catalyst, in reduced form, promotes the following reactions: $2\,CO + O_2 \rightarrow 2\,CO_2$ and $2\,H_2 + O_2 \rightarrow 2\,H_2O$. The catalyst is spread over two columns (no. 21), each filled with 1 kg. The columns are made of glass. The catalyst is visible and is partially insulated by a 2-cm-thick, glass-wool blanket (no. 23) surrounded by an aluminium sheet cylinder (no. 24). A black color indicates that the copper is reduced, which is the required condition. In the case of an excess of air, the catalyst becomes green. Then the gas-to-air ratio has to be readjusted. The catalyst is heated by a 135-W electrical element (no. 22), placed in the core of the column. The normal temperature of the columns is 230 C. When the inert gas leaves the catalyst columns, the O_2 content is reduced to less than 1 μliter/liter, measured with an oxygen traces analysator type Elcoflux C 5 (Hartmann und Braun, Germany). The gas is then ready for use.

Glove box. The glove box is made of sheet steel (1.5 mm thickness) and has a glass window at the oblique upper side. The dimensions are as follows: length, 150 cm; depth, 75 cm; and height, 50 cm. In the front side three gloves (butyl rubber) are installed. With use of the glove on the right, the airlocks can be opened.

The box is equipped with two round steel airlocks to bring materials in and out (35 cm long, 30 cm in diameter; and 17 cm long, 10 cm in diameter, respectively). On the top of each airlock are two valves and a vacuum gauge. One valve is connected with a vacuum pump (Cenco, Pressovac) and one with the inert gas supply. To introduce materials into the box, the lock is evacuated and refilled with inert gas twice. The entire process of introducing materials can be completed in a few minutes.

The box is purged continuously with the inert gas at such a rate as to replace the atmosphere every 30 min. The gas leaves the box via a gas trap, securing a small overpressure in the box. An electrical lead is introduced into the box via a rubber stopper to feed the lighting and four plugs.

The equipment in the box comprises the routine equipment for conventional bacteriological work, such as an incubator, loops, electrical heating element, diluting tubes, mixer, etc. The temperature in the box is equal to the room temperature (22 C).

Media. We have used the agar medium described by Wensinck and Ruseler-van Embden (10), to which 7% sheep blood was added. As diluting medium we

used the same medium without agar and sheep blood. Immediately after sterilization the media were introduced into the box, and next the vitamin mixture as well as the sheep blood were added. The agar medium was poured into plastic petri dishes inside the glove box. The plates were used after being prereduced for 24 h. The incubation time was 1 week.

Oxidation-reduction potential. It is possible to connect electrodes inside the box to measure the oxidation-reduction (O-R) potential in agar media. For this purpose we used a standard calomel reference electrode, a platinum wire electrode, and a radiometer Copenhagen type 22. O-R potentials in agar media were measured by pressing the electrodes into the agar. The readings are corrected for the voltage of the calomel electrode, to give the E_h.

Mice. The animals used were CRF mice (9) kept under germ-free conditions. These animals are colonized with a purely anaerobic intestinal flora (10). The mice were killed by cervical dislocation and immediately thereafter were introduced into the glove box. The cecum was then opened under aseptic conditions and 0.05 g of the contents was taken and homogenized in 5 cm^3 diluting medium by means of an Ultra-turrax type TP 10 N (Janke und Kunkel K. G., Germany). A number of tenfold dilutions were made by means of dosing syringes. From a 10^{-9} dilution, 0.1 cm^3 was placed on the plates by means of a dosing syringe and afterwards spread over the plates. Besides this procedure, conventional anaerobic techniques, with the same dilutions, (anaerobic jars, with a Gas-Pak catalyst) were used. From the dilutions used, direct microscope clump counts were determined (4).

RESULTS

O-R potential in agar media. The media show varying E_h values just after introduction into the glove box (−200 to −250 mV). After 24 h, however, a constant value is reached: −250 to −300 mV at a pH of 7.

Cultivation of cecum bacteria. With anaerobic jars it is possible to cultivate 1.3 to 5.8% of the total count (Table 1). In general, only two species are found: a gram-positive, semicircular sporeforming rod and a gram-positive, straight or curved sporeforming rod. In the glove box we were able to cultivate a higher percentage, i.e., 17.7 to 37.1% of the total counts (Table 1).

In the glove box it was possible to cultivate a number of strictly anaerobic species from the cecum contents of CRF mice, besides the two species mentioned above. These anaerobic species were as follows. (i) A short gram-negative tapered rod in chains (Fig. 2). Colony type: 2 mm, round, convex, yellow. (ii) A gram-negative rod with rounded ends, sometimes curved (Fig. 3.). Colony type: 3 mm, irregular, flat, grey. (iii) A short, gram-negative, often bipolar stained rod (Fig. 4.) Colony type: 0.5 mm,

TABLE 1. *Total counts of bacteria cultured by two methods from mouse cecum compared with direct microscope counts*

Mice no.	Direct microscope clump counts	Cultivated in McIntosh-jar	Cultivated in glove box
1	3.5×10^{10}	4.7×10^{8} (1.3)[a]	8.4×10^{9} (24.0)
2	3.3×10^{10}	1.8×10^{9} (5.4)	9.0×10^{9} (27.3)
3	2.6×10^{10}	1.2×10^{9} (4.6)	6.5×10^{9} (25.0)
4	2.6×10^{10}	1.5×10^{9} (5.8)	4.6×10^{9} (17.7)
5	2.8×10^{10}	9.0×10^{8} (3.2)	6.3×10^{9} (22.5)
6	3.5×10^{10}	1.1×10^{9} (3.1)	1.3×10^{10} (37.1)

[a] Numbers in parentheses indicate percentages.

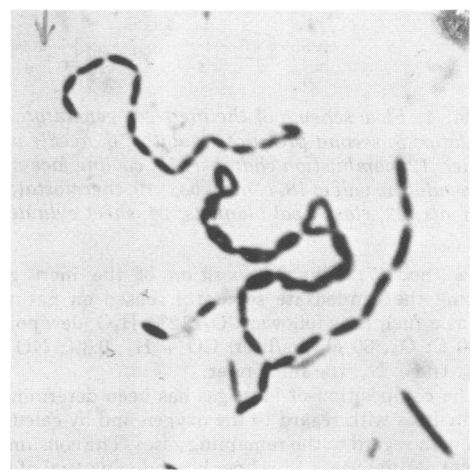

FIG. 2. *Species 1 (×1,536). Gram-stained film of 1-week culture.*

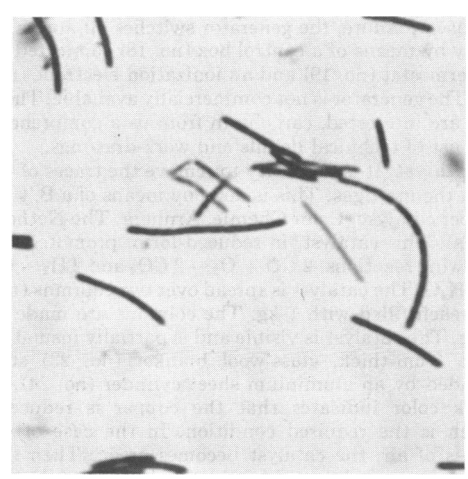

FIG. 3. *Species 2 (×1,536). Gram-stained film of 1-week culture.*

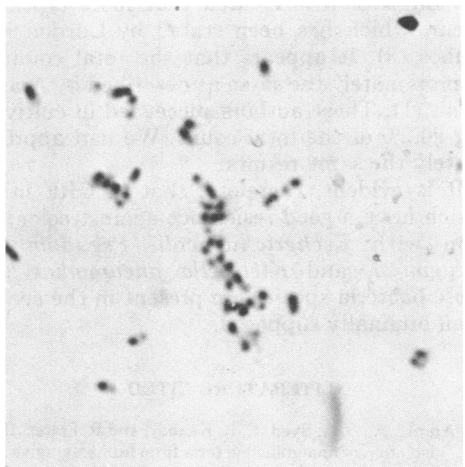

FIG. 4. *Species 3 (×1,536). Gram-stained film of 1-week culture.*

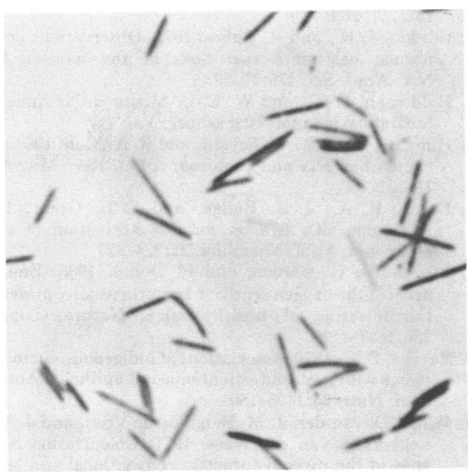

FIG. 5. *Species 4 (×1,536). Gram-stained film of 1-week culture.*

round, convex, white. (iv) A gram-positive tapered rod (Fig. 5.). Colony type: 2.5 mm, round, convex, brown. (v) A large, gram-negative tapered rod (Fig. 6). Colony type: greyish swarming. (vi) A slight curved rod (Fig. 7.). Colony type: 4 mm, round, convex, grey.

DISCUSSION

It is possible to maintain an anaerobic atmosphere in a glove box continuously by purging the box with O_2-free gas.

To make this system economically acceptable it is required that the gas is available in large amounts and at low cost. This could be achieved by using the combustion products of natural gas.

The advantages of two earlier systems, mentioned in the introduction, are combined in this system. The principal advantages are as follows. (i) A constant composition of the atmosphere is maintained. (ii) The equipment in the box is kept under permanent anaerobic conditions. (iii) No preparation time before working in the box is needed. (iv) A supply from gas-cylinders is not necessary. (v) The media are incubated in the working space and can be controlled at any time. (vi) Periodic regeneration of the catalyst is not needed. (vii) Poisoning the catalyst with toxic gases produced by the bacteria is not possible. (viii) There is no need to remove water vapor.

A disadvantage of our system, however, is that in case of interruption of supply of water, electricity, or natural gas, the apparatus

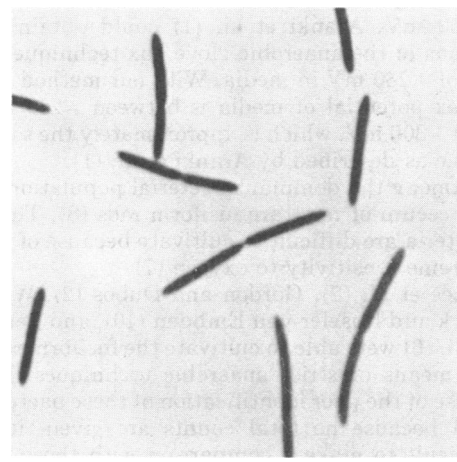

FIG. 6. *Species 5 (×1,536). Gram-stained film of 1-week culture.*

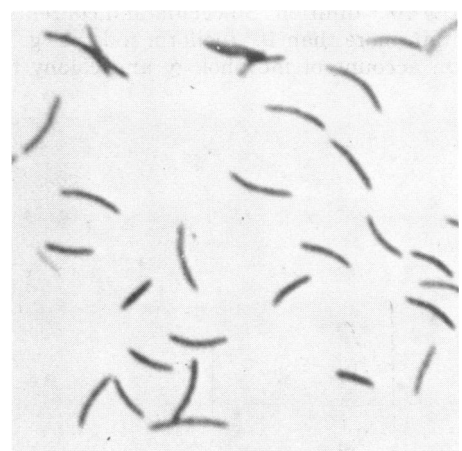

FIG. 7. *Species 6 (×1,536). Gram-stained film of 1-week culture.*

switches off. In the 9 months that we have used the apparatus there was only one brief failure of electricity.

An objection may be that we do not know the influence on bacterial growth of traces of CO, NO, and NO_2 that may be present in the inert gas. However, we have not found any indication of a harmful influence.

The measured values of the O-R potential in media are equal to those described by Aranki et al. (1). The values are achieved after 1 day of prereducing.

It is difficult to compare different anaerobic methods if the same material is not used. On the other hand the redox potential of media can be compared with the results obtained by other authors. Hungate et al. (5) were able to cultivate the fastidious strict anaerobic methanogenic bacteria in media with an E_h lower than -150 mV. Aranki et al. (1) could obtain by means of the anaerobic glove box technique an E_h of -280 mV in media. With our method the redox potential of media is between -250 mV and -300 mV, which is approximately the same value as described by Aranki et al. (1).

Among the dominant bacterial population in the cecum of mice are fusiform rods (8). These bacteria are difficult to cultivate because of the extreme sensitivity to oxygen (7).

Lee et al. (7), Gordon and Dubos (2), Wensinck and Ruseler-van Embden (10), and Leach et al. (6) were able to cultivate the fusiform rods by means of strict anaerobic techniques. Because of the poor identification of these bacteria and because no total counts are given, it is difficult to make a comparison with these authors.

Our investigation showed that it is possible to cultivate fusiform rods and that they occurred at the 10^{-9} dilution. So cecum and contents do contain more than 10^9 fusiform rods per g.

On account of morphology and colony type the impression is created that more types do occur, which has been stated by Gordon and Dubos (3). It appears that the total count is approximately the same as described by Aranki et al. (1). These authors succeeded in cultivating 20.2% of the total count. We had approximately the same results.

It is evident, therefore, that in CRF mice, which have a good resistance against colonization with *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* (9), more bacteria species are present in the cecum than originally supposed.

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