J. Hyg., Camb. (1971), **69**, 413
With 2 plates
Printed in Great Britain

The intestinal flora of colonization-resistant mice

By F. WENSINCK and J. G. H. RUSELER-VAN EMBDEN
Laboratory of Medical Microbiology, Medical Faculty, Rotterdam, The Netherlands

(*Received* 1 *March* 1971)

SUMMARY

To facilitate the isolation of anaerobes from cultures in which swarming organisms were present Roux flasks were adapted to provide anaerobic conditions. Using these flasks, five species of anaerobes were isolated from the caecum of mice resistant to colonization with *Escherichia coli* and other Gram-negative organisms. These species accounted for the majority of organisms seen in films of caecal material. No aerobes were isolated and the flora of these mice apparently consisted of a limited number of anaerobes representing part of the intestinal flora of conventional mice. Three of the species were clostridia and another was identified as *Propionibacterium acnes*. One species resembled *Catenabacterium* but was not identified. One of the *Clostridium* species could only be isolated when the medium was supplemented with yeast extract and vitamins. This species sporulated on media with ammonium phosphate as the source of nitrogen; tryptone completely suppressed sporulation.

INTRODUCTION

When mice colonized with a purely anaerobic intestinal flora were challenged by oral administration of graded doses of *Escherichia coli* and other Gram-negative organisms, colonization resistance (CR) was observed, i.e. the bacteria were eliminated from the intestinal tract in periods of time related to the challenge dose. These mice were the offspring of germ-free mice contaminated with the intestinal flora of a conventional mouse treated with antibiotics and were called CRF-mice. The effects of oral administration of antibiotics on the intestinal flora of conventional mice and the resulting changes of CR were studied by van der Waaij, Berghuis-de Vries & Lekkerkerk-van der Wees (1971). The present paper deals with the intestinal flora of CRF-mice. By examination of Gram-stained films of caecal contents the following organisms were recognized: (*a*) Gram-positive, semi-circular rods, almost exclusively arranged in clusters; (*b*) Gram-positive straight rods, occasionally with oval, terminal spores; (*c*) Gram-positive, irregularly stained rods and (*d*) tapered rods. Many tapered rods were large, cigar-like organisms and others very slender, Gram-negative fusiform rods. Large tapered rods were Gram-positive or negative and some showed demarcated Gram-positive areas. In view of the morphological differences between large, Gram-variable and slender Gram-negative rods it seemed likely that the population of tapered rods consisted of at

least two species. From these observations it was concluded that at least five species were represented in the intestinal flora.

With conventional anaerobic techniques, a *Clostridium* species was isolated but spreading growth of tapered rods interfered with the isolation of other organisms. A method was therefore devised to facilitate the early recognition of colony formation. Essentially, it consisted in using Roux flasks adapted to provide anaerobic conditions. Using these flasks, five species of anaerobes were isolated. One of them, a large, Gram-labile tapered rod, was often present in primary cultures but disappeared on subculture. This suggested that growth depended on metabolites of one or more of the other organisms. Although extracts from primary cultures of caecal material were growth-promoting, no effort was made to isolate the active principle since a vitamin supplement was much more effective. Eventually, pure cultures were obtained on synthetic media supplemented with yeast extract and vitamins. In the present paper the method of anaerobic cultivation is described and the characteristics of the species isolated are reported.

MATERIALS AND METHODS

Mice

Groups of five CRF-mice housed in an isolation cage (van der Waaij, 1968) were received from the Radiobiological Institute TNO (Rijswijk, The Netherlands). The animals were killed with ether; a small piece of the caecal wall was cut out and put immediately on the medium in culture flasks. The material was distributed over the surface with spreaders made by bending Pasteur pipettes at right angles, the short limb being 1 cm. long.

Culture flasks

The conical neck of Pyrex-brand Roux flasks (19 × 10 × 5 cm.) was ground to fit the B 29 standard joint adapter. At a distance of about 2 cm. from the neck a socket fitting the B 14½ joint adapter was attached to the upper part of the side wall of the flask. Flasks were also modified by folding the wall with a triangular mould to form a transversal dam separating two medium compartments (Pl. 1, fig. 1). Stopcocks and adapters were lubricated with high-vacuum grease (Glisseal; PMC, Solothurn, Switzerland). The side stopcock served to introduce gas into the flask; with the central stopcock removed the interior was easily accessible to manipulation with Pasteur pipettes.

Anaerobic conditions

A commerical mixture of 5 % CO_2 in nitrogen passing through an oil lock filled with mineral oil was freed from oxygen by leading it through a column of reduced BTS catalysator (Color Chemie, Arnhem, The Netherlands) heated at about 70° C. The columns were of the type described by Drasar (1967) and Moore (1966) and contained about 500 g. of the catalysator, which was activated at intervals by passing hydrogen through the column. Before flasks were connected to the gas supply, gas was allowed to stream out freely for 2 min. The side stopcock was connected to the tubing (butane-gas tubing, Vredestein) and opened. Immediately

afterwards, the central stopcock was opened and the gas flow adjusted with the reducing valve of the gas cylinder to maintain slight pressure at the opening of this stopcock; the central stopcock adapter was then removed. When closing a flask, this adapter was replaced and gas allowed to stream through the flask for a few minutes. The stopcocks were then closed simultaneously. To prevent the adapters being forced out by gas produced by the culture the inside pressure was slightly reduced before placing flasks in the incubator. Resazurin (2 mg./l.) was added to all media as an indicator to show that anaerobiosis was maintained.

Isolation media

The medium of Hamilton & Zahler (1957) was used, with Oxoid No. 3 agar substituted for Difco agar. This medium was suited for the isolation and propagation of the intestinal anaerobes with the exception of large, Gram-labile tapered rods. These organisms were isolated on the following medium: tryptone (Oxoid), 10 g.; glucose, 1 g.; soluble starch, 4 g.; yeast extract, 5 g.; $K_2HPO_4.2H_2O$, 2 g.; KH_2PO_4, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; NaCl, 5 g.; cysteine HCl, 0·5 g.; distilled water 1 l. The pH was adjusted to 7·2 and 1·5 % agar was added. To the sterilized medium, the following vitamin solution was added (10 ml./l.): *p*-aminobenzoic acid, 100 mg.; biotin, 0·03 mg.; calcium pantothenate, 1·2 g.; folic acid, 100 mg.; niacin, 100 mg.; pyridoxal phosphate, 100 mg.; riboflavin, 100 mg.; thiamine HCl, 100 mg.; distilled water 1 l. The solution was adjusted to pH 6·8 and sterilized by filtration.

Sterilization of the isolation media

The media were prepared in culture flasks and sterilized in pressure cookers for 10 min. at 100° C. The cookers were allowed to cool to about 60° C. and the pressure was maintained at 1 atmosphere by introducing the gas mixture at intervals. The cookers were opened and the flasks connected to the gas supply immediately. Gas was allowed to stream through the flasks until the media had solidified. The vitamin solution was introduced into the flask when the medium had cooled to about 45° C.

Sporulation medium

This medium contained per litre of distilled water: $(NH_4)_2HPO_4$, 3 g.; glucose, 3 g.; NaCl, 4 g.; KCl, 1 g.; $MgSO_4.7H_2O$, 0·5 g.; yeast extract, 3 g.; cysteine HCl, 0·5 g. The pH was adjusted to 7·2 and 0·5 % agar was added. The medium was sterilized in the same way as the isolation media and the vitamin solution was added to the cooled medium.

Fermentation media

The Hamilton and Zahler medium was prepared with glucose omitted and 0·3 % agar added and was sterilized at 120° C. for 15 min. Concentrated carbohydrate solutions were sterilized by filtration and added to the basal media to give a final concentration of 0·5 %. The following carbohydrates were tested: arabinose, xylose; fructose, galactose, glucose, mannose; lactose, maltose, saccharose, trehalose; starch; glycerol, mannitol. The media were distributed in tubes which were placed in a boiling water bath for 20 min. and cooled before being inoculated.

Fermentation products

The pH of 1-week-old cultures on fermentation media and on the corresponding basal medium was determined potentiometrically; a difference of at least 0·5 pH unit was considered evidence of carbohydrate fermentation. Cultures grown for 1-week in an isolation medium with agar omitted were centrifuged and the supernatants analysed for lactic acid (Long, 1947) and for volatile acids by a modification of the method outlined by Skerman (1967). This method involves the extraction of volatile acids from a distillate of the acidified culture with ether. This step was modified according to Guillaume & Osteux (1955). The distillate was brought to pH 9·0 with 1 N-Na_2CO_3 and evaporated to dryness under reduced pressure at 50° C. on a rotary evaporator. The residue was dissolved in water and treated with Zeocarb 225 (British Drug Houses); the acids were then converted to their ammonium salts by addition of 1 N-NH_4OH.

Miscellaneous tests

Standard methods were used to demonstrate catalase, gelatinase, hydrogen sulphide and indole production and reduction of nitrate to nitrite (Cruickshank, 1968).

RESULTS

Primary cultures of caecal contents on the Hamilton and Zahler medium consisted of colonies and areas of spreading growth. By subculturing on this medium three morphologically different organisms forming characteristic colonies were easily obtained in pure culture. Spreading growth consisted mainly of slender, Gram-negative fusiform rods which were obtained in pure culture by passage on thoroughly dried media solidified with 2% agar. From a comparison of several strains of these four types of organisms isolated from different groups of mice it was concluded that they represented four well-defined species provisionally called A, B, C and D. Their properties are summarized below in the same order as the organisms are mentioned in the Introduction. The isolation of a fifth species (E), a large, Gram-labile tapered rod, was difficult and is therefore described in detail. The five species were strictly anaerobic and no growth was obtained when caecal contents were incubated aerobically.

Species A

Morphology. In films of caecal material the organisms are arranged in clusters of Gram-positive semicircular rods and the same arrangement is seen in young cultures (Pl. 1, fig. 2), where the clusters are embedded in large masses of capsular material. In older cultures the organism is highly pleomorphic, Gram-negative with Gram-positive granules and shows true branching.

Surface colonies. Circular, umbonate, white, mucous, 0·5–2·0 mm.

Physiology. Strictly anaerobic, catalase negative. Acid but no gas produced from carbohydrates (Table 1); glucose fermented with the production of acetic acid. Gelatinase, hydrogen sulphide and indole not produced, nitrate not reduced.

Identification. Morphologically, this organism closely resembles 'doughnut-shaped' rods isolated from the mouse caecum by Gordon & Dubos (1970) and identified as *Catenabacterium catenaforme* or *C. contortum*. True branching seems to exclude species A from *Catenabacterium*; it could not be assigned to one of the other rather ill-defined genera in which Gram-positive non-sporulating anaerobic rods are placed.

Table 1. *Fermentation of carbohydrates by species A–E*

	Species				
	A	B	C	D	E*
Substrate					
Arabinose	−	+	−	+	.
Xylose	−	−	−	+	.
Fructose	+	+	+	+	.
Galactose	+	+	+	+	.
Glucose	+	+	+	+	+
Mannose	+	+	+	+	.
Lactose	+	−	−	+	−
Maltose	+	+	−	−	+
Saccharose	+	+	−	+	+
Trehalose	−	+	+	−	.
Glycerol	−	−	+	−	.
Mannitol	−	+	−	−	.
Soluble starch	−	+	−	−	−
Products of glucose fermentation					
Acetic acid	+	+	+	+	+
Butyric acid	−	+	−	+	+
Lactic acid	−	−	−	−	−
Propionic acid	−	−	+	−	+
Valeric acid	−	−	−	−	+
Gas	−	+	−	+	−
Final pH	5·6	4·9	5·5	4·9	5·3

* Fermentation pattern incompletely studied with media in culture flasks.

Species B

Morphology. Straight or slightly curved rods, 0·5 × 4–5 μ, occurring singly; Gram-positive in young cultures, non-motile, non-capsulated. Spores are oval, terminal and slightly wider than the diameter of the rod.

Surface colonies. Circular, convex, white, smooth, 1–3 mm.

Physiology. Strictly anaerobic, catalase negative. Acid and gas produced from carbohydrates (Table 1); glucose fermented with the production of acetic and butyric acids and carbon dioxide. Gelatinase, hydrogen sulphide and indole not produced, nitrate not reduced.

Identification. The organism is considered to be a *Clostridium* not belonging to one of the well-defined species.

https://doi.org/10.1017/S0022172400021665 Published online by Cambridge University Press

Species C

Morphology. Gram-positive, irregularly stained, pleomorphic rods, occurring singly, in pairs or in groups and showing the arrangement of corynebacteria. Non-motile, non-capsulated.

Surface colonies. Irregular, umbonate, lobate, 1–4 mm., coloured pink after 3–5 days.

Physiology. Anaerobic when isolated but aerotolerant after a few subcultures; catalase positive. Acid but no gas produced from carbohydrates (Table 1); glucose fermented with the production of acetic and propionic acids. Acetic and propionic acids produced from lactate. Gelatin hydrolysed, hydrogen sulphide not produced, indole positive and nitrate reduced to nitrite.

Identification. The organism was identified as *Propionibacterium acnes* (*Corynebacterium acnes*).

Species D

Morphology. Gram-negative, tapered rods, $0{\cdot}5 \times 3$–$10\ \mu$, occurring singly and in pairs (Pl. 1, fig. 3). Motile by numerous peritrichate flagella; motility stops soon after exposure to air. Non-capsulated. Spores are not readily formed and are oval, subterminal and hardly wider than the rods.

Surface colonies. Spreading growth, translucent with irregular light reflexion. Round, white colonies, 1–2 mm., are formed on thoroughly dried media solidified with 2% agar.

Physiology. Strictly anaerobic, catalase negative. Acid and gas produced from carbohydrates (Table 1); glucose fermented with the production of acetic and butyric acids and carbon dioxide. Gelatinase negative, hydrogen sulphide and indole produced, nitrate not reduced.

Identification. Morphologically, this organism closely resembles fusobacteria and has several characters in common with the medium and thin tapered rods isolated from the mouse caecum by Gordon & Dubos (1970) and assigned by them to *Fusobacterium*. Species D differs in many respects from this genus as characterized by Baird-Parker (1960) and Barnes & Goldberg (1968). The cells are motile by peritrichate flagella, carbohydrates are actively fermented with copious gas production, the terminal pH value of glucose-fermenting cultures is low and pentoses are fermented. Moreover, species D grows well on simple media like peptone water with 0·3% agar. It could be assigned to *Clostridium* when sporulation was demonstrated on media with 0·15% fructose. Only a few percent of the cells sporulate.

Species E

Morphology. Tapered cigar-shaped rods, Gram-positive in young cultures, 1×9–$15\ \mu$. Cells of 24 hr. old cultures are either Gram-negative or show band-like Gram-positive areas (Pl. 2, fig. 4). Motile by numerous peritrichate flagella; motility stops immediately after exposure to air. When young cultures are observed with a pocket lens, irregular patterns of reflected light are seen which change con-

tinuously as a result of cellular movement. Spores are not readily formed and are subterminal, cylindrical, conical and do not swell the cells (Pl. 2, fig. 5). Non-capsulated.

Surface colonies. Spreading growth with irregular light reflexion; on dry media, irregular-shaped colonies, 1–3 mm., are formed within 24–48 hr. The heaped cell material is rather sticky and slightly brown-coloured.

Physiology. Strictly anaerobic, catalase negative, and extremely sensitive to air unless cultivated on the sporulation medium. Acid but no gas produced from carbohydrates (Table 1). Glucose fermented with the production of propionic and acetic acids and small quantities of butyric and valeric acids. Gelatinase negative, abundant production of hydrogen sulphide, indole negative. Preliminary studies of growth requirements have shown that even in the presence of the vitamin supplement growth is improved by yeast extract. Sporulation is completely suppressed by tryptone. On media with the ammonium ion as the source of nitrogen sporulation starts after 2–3 days; only a few per cent of the cells sporulate.

Isolation. After many unsuccessful attempts to isolate this species it was found that it is essential that vitamins be added to the medium. Moreover, the glucose concentration has to be low (0·1 %); otherwise, species E is readily overgrown. In primary cultures of caecal material the organism mutiplies in areas of spreading growth together with species D. After two or three subcultures these areas mainly consist of species D and E; subcultures are then made on the isolation medium with 0·5 % glucose and with soluble starch omitted. Species D and E grow rapidly on this medium and the mixed culture is subcultured on the sporulation medium. When a reasonable number of spores has formed (3–4 days), growth is harvested, suspended in NaCl solution (0·9 %), heated for 10 min. at 70° C. and plated out on the isolation medium with 0·5 % glucose. The resulting culture is likely to consist of species E only.

Identification. The organism is considered to be a *Clostridium* not belonging to one of the well-defined species.

DISCUSSION

It is generally agreed that methods for isolating fastidiously anaerobic bacteria should fulfil the following requirements: (*a*) the gas mixture should be freed from oxygen, (*b*) the media should be reduced before they are inoculated, and (*c*) exposure to air of material to be cultivated must be minimal (Drasar, 1967; Aranki, Syed, Kenney & Freter, 1969; Moore, Cato & Holdeman, 1969; Gordon & Dubos, 1970). The method described meets these requirements. By sterilizing the media in pressure cookers and introducing the gas mixture into the cookers while cooling and into the flasks while the media solidify, oxidation is sufficiently prevented as indicated by the absence of coloration by oxidized resazurin. The redox potential increases when vitamin solutions are mixed with the sterilized medium but the resulting pink colour disappears within a few minutes.

We consider the anaerobic flask to be useful for separating anaerobes in mixed cultures. By removing condensed water and passing the gas mixture for about 20 min. through the flasks the medium surface can be made sufficiently dry to

limit swarming – a property displayed by several anaerobic species. Moreover, the flasks can be inspected at any time and individual colonies can thus be recognized early and subcultured on the separate medium compartment of the same flask or on other flasks to which material can be transferred within one second.

The species isolated account for the majority of the organisms seen in films of caecal contents. Therefore, it seems reasonable to conclude that most if not all of the species present were cultivated. Evidently, this flora is different from the anaerobic intestinal flora of man and conventional mice in which anaerobic lactobacilli (bifidobacteria) and *Bacteroides* species predominate (Schaedler, Dubos & Costello, 1965; van Houte & Gibbons, 1966; Drasar, 1967; Lee, Gordon & Dubos, 1968; van der Wiel-Korstanje & Winkler, 1970). These organisms were not isolated from the mice studied. Instead, their intestinal flora is dominated by two species of tapered rods in which sporulation was eventually demonstrated. One of these *Clostridium* species (species E) outnumbers other organisms in films of caecal wall mucus, where it apparently multiplies. It closely resembles tapered rods shown to be present in the intestine of mice (Lee *et al.* 1968) and ground squirrels (Barnes & Burton, 1970), but as far as is known it has not been isolated and characterized before. A preliminary survey indicates that this *Clostridium* species is a member of the intestinal flora of conventional laboratory rodents. Moreover, results from experiments with germ-free mice contaminated with various combinations of the five species described strongly suggest that colonization resistance is due to the presence of this *Clostridium*.

We would like to thank Miss P. H. J. Kustermans for skilful technical assistance. Sporulation in species D was demonstrated by Mr J. M. J. C. Scheres (Experimental Gerontology Unit TNO, Rijswijk, The Netherlands).

REFERENCES

ARANKI, A., SYED, S. A., KENNEY, E. B. & FRETER, R. (1969). Isolation of anaerobic bacteria from human gingiva and mouse cecum by means of a simplified glove box procedure. *Applied Microbiology* **17**, 568.

BAIRD-PARKER, A. C. (1960). The classification of Fusobacteria from the human mouth. *Journal of General Microbiology* **22**, 458.

BARNES, E. M. & BURTON, G. C. (1970). The effect of hibernation on the caecal flora of the thirteen-lined ground squirrel (*Citellus tridecemlineatus*). *Journal of Applied Bacteriology* **33**, 505.

BARNES, E. M. & GOLDBERG, H. S. (1968). The relationship of bacteria within the family Bacteroidaceae as shown by numerical taxonomony. *Journal of General Microbiology* **51**, 313.

CRUICKSHANK, R. (ed.) (1968). *Medical Microbiology*, 11th ed. Edinburgh: E. and S. Livingstone Ltd.

DRASAR, B. S. (1967). Cultivation of anaerobic intestinal bacteria. *Journal of Pathology and Bacteriology* **94**, 417.

GORDON, J. H. & DUBOS, R. (1970). The anaerobic bacterial flora of the mouse cecum. *Journal of Experimental Medicine* **132**, 251.

GUILLAUME, J. & OSTEUX, R. (1955). Détermination des acides aliphatiques volatils de C_1 à C_6 dans les liquides biologiques par chromatographie sur papier. *Comptes Rendus de l'Académie des Sciences (Paris)* **241**, 501.

HAMILTON, R. D. & ZAHLER, S. A. (1957). A study of *Leptotrichia buccalis*. *Journal of Bacteriology* **73**, 386.

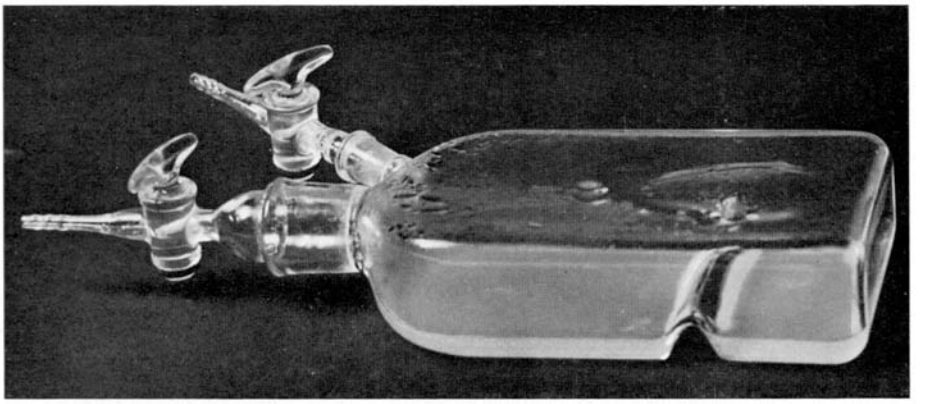

Fig. 1

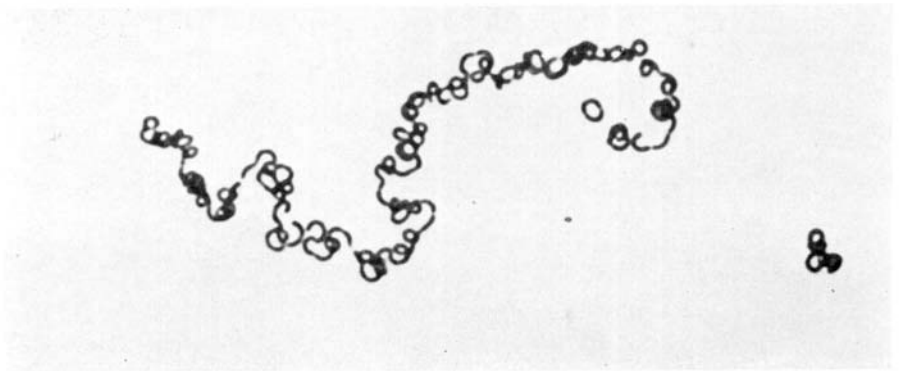

Fig. 2

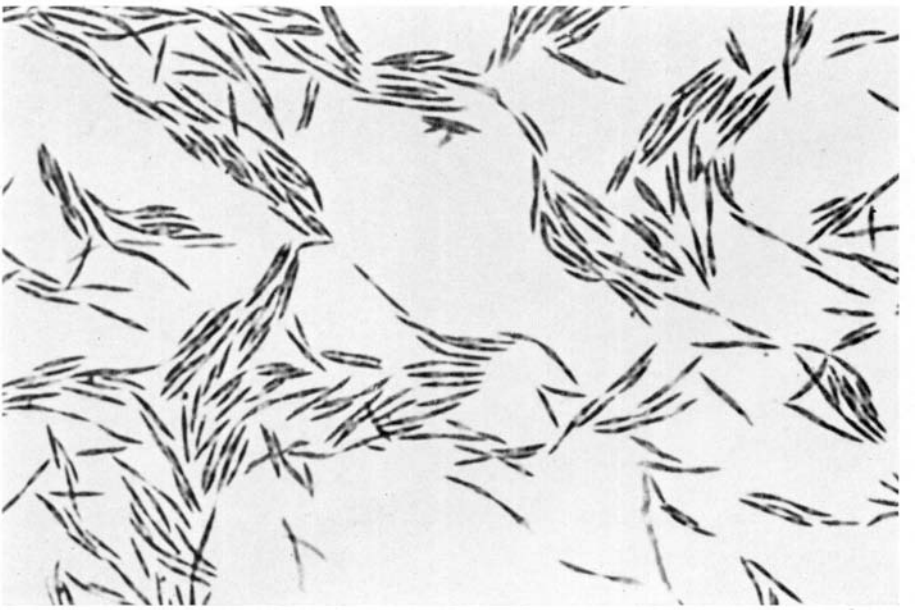

Fig. 3

F. WENSINCK AND J. G. H. RUSELER-VAN EMBDEN

(*Facing p.* 420)

https://doi.org/10.1017/S0022172400021665 Published online by Cambridge University Press

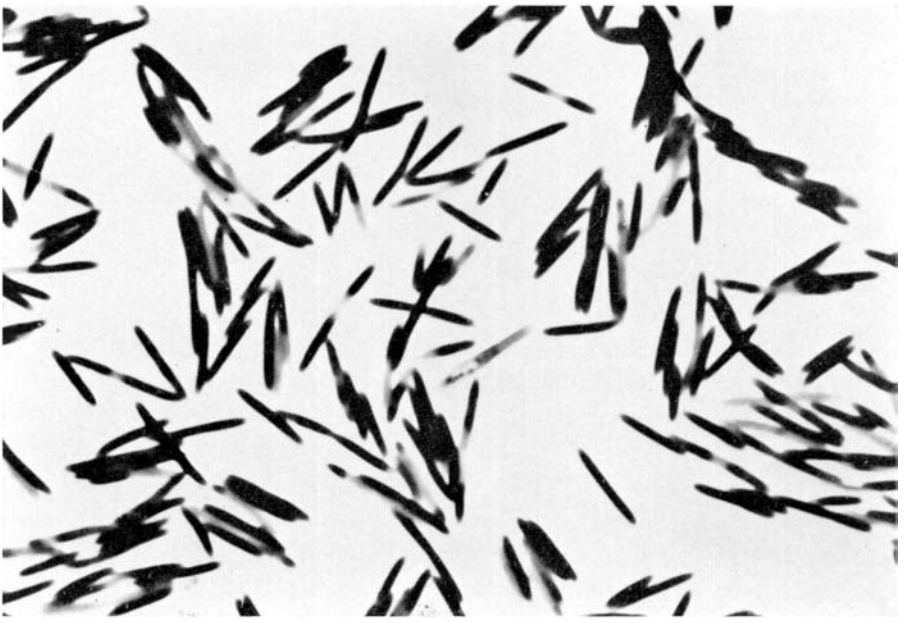

Fig. 4

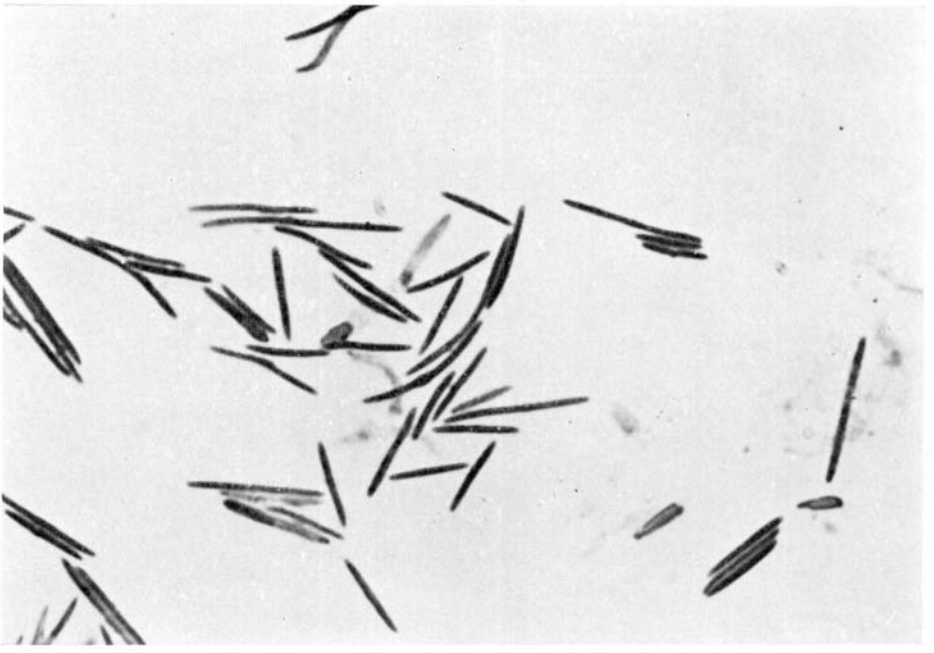

Fig. 5

https://doi.org/10.1017/S0022172400021665 Published online by Cambridge University Press

HOUTE, J. VAN & GIBBONS, R. J. (1966). Studies of the cultivable flora of normal human feces. *Antonie van Leeuwenhoek Journal of Microbiology and Serology* **32**, 212.

LEE, A., GORDON, J. & DUBOS, R. (1968). Enumeration of the oxygen sensitive bacteria usually present in the intestine of healthy mice. *Nature, London* **220**, 1137.

LONG, C. (1947). The stabilization and estimation of lactic acid in blood samples. *Biochemical Journal* **40**, 27.

MOORE, W. E. C. (1966). Techniques for routine culture of fastidious anaerobes. *International Journal of Systematic Bacteriology* **16**, 173.

MOORE, W. E. C., CATO, E. P. & HOLDEMAN, L. V. (1969). Anaerobic bacteria of the gastrointestinal flora and their occurrence in clinical infections. *Journal of Infectious Diseases*, **119**, 641.

SCHAEDLER, R. W., DUBOS, R. & COSTELLO, R. (1965). Association of germfree mice with bacteria isolated from normal mice. *Journal of Experimental Medicine* **122**, 77.

SKERMAN, V. B. D. (1967). *A Guide to the Identification of the Genera of Bacteria*, 2nd ed. Baltimore: Williams and Wilkins.

WAAIJ, D. VAN DER (1968). The persistent absence of Enterobacteriaceae from the intestinal flora of mice following antibiotic treatment. *Journal of Infectious Diseases* **118**, 32.

WAAIJ, D. VAN DER, BERGHUIS-DE VRIES, J. M. & LEKKERKERK-VAN DER WEES, J. E. C. (1971). Colonization resistance of the digestive tract in conventional and antibiotic treated mice. *Journal of Hygiene* **69**, 405.

WIEL-KORSTANJE, J. A. A. VAN DER & WINKLER, K. C. (1970). Medium for differential count of the anaerobic flora in human feces. *Applied Microbiology* **20**, 168.

EXPLANATION OF PLATES

PLATE 1

Fig. 1. Roux flask adapted to anaerobic cultivation.

Fig. 2. Species A. Gram-stained film of 24 hr. culture (× 1400).

Fig. 3. Species D. Gram-stained film of 24 hr. culture (× 1400)

PLATE 2

Fig. 4. Species E. Gram-stained film of 12 hr. culture (× 1400).

Fig. 5. Species E. Malachite green-safranine stained film of 3-day-old culture on sporulation medium (× 1400).