***Eubacterium fissicatena* sp.nov. Isolated from the Alimentary Tract of the Goat**

By MARGARET M. TAYLOR

The Hannah Research Institute, Ayr, Scotland

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SUMMARY

A new species of *Eubacterium* has been isolated from the rumen and caecum of fistulated goats. This species, *Eubacterium fissicatena*, degraded the ribityl chain of riboflavin hydroxyethylflavin.

INTRODUCTION

The isolation of hydroxyethylflavin (HEF) as a bacterial degradation product of riboflavin was first reported by Miles & Stadtman (1955), who used a partly purified culture of soil bacteria grown with excess riboflavin under anaerobic condition. HEF can be obtained from the urine of man after the ingestion of large doses of riboflavin (West & Owen, 1969). HEF also occurs in the milk and urine of sheep and goats and the urine of ruminating kids and calves that are fed riboflavin, but not if the riboflavin is injected subcutaneously (Owen & West, 1970). HEF can be isolated from the caecum of normal but not germ-free rabbits (Owen, West & Coates, 1970), which provides further evidence that HEF is a bacterial degradation product. In the present work, a bacteriological examination was made of samples from fistulated goats of rumen and caecal contents used for the chemical study of the bacterial enzyme system responsible for riboflavin degradation.

Eubacterium can be isolated from soil and from a wide variety of intestinal sources such as those of man, marine fish, sheep, foals (*Bergey's Manual of Determinative Bacteriology*, 1957) and cows (Bryant & Robinson, 1962). A new species of *Eubacterium* is described which has been isolated from the rumen and caecum of the goat and which degrades riboflavin to HEF.

METHODS

Samples were obtained from fistulated goats. The particulate matter and protozoa were removed from rumen samples by filtration and differential centrifugation and the bacterial deposit was inoculated into various media with excess riboflavin. Caecal samples were inoculated directly into the media. The most satisfactory fluid medium for the production of hydroxyethylflavin (HEF) contained 1.5% Oxoid proteose-peptone, 0.25% Oxoid liver digest, 0.5% Oxoid yeast extract and 1.0% NaCl (YE broth) with about 0.1% riboflavin (RYE broth). The medium was sterilized at 121 °C for 15 min. A hot iron nail, as a reducing agent, was added to each tube just before inoculation. Oxoid blood agar base no. 2 with the addition of 0.5% NaCl (YE agar) was used as a solid medium; riboflavin was added when required (RYE agar). The riboflavin-enriched mixed cultures were plated on RYE agar. Single colonies were inoculated into RYE broth. After 24 to 48 h of incubation, the broths were tested for HEF production. Tubes giving a positive reaction were plated for purity and further colonies tested for HEF production. This was continued until a pure culture had been obtained. HEF-producing colonies were identified by the orange colour of the

oxidized HEF. During isolation, the solid media did not contain a reducing agent. When pure cultures had been obtained, 2 g reduced iron powder (British Drug Houses Ltd, Poole, Dorset), suspended in 10 ml YE agar, was used in the lower layer of a double-layer YE plate. The cultures were incubated under hydrogen in an anaerobic jar at 37 °C.

Eubacterium ruminantium (GAI95, B₁C23), *E. limosum* (*Butyribacterium rettgeri*) (L34) were obtained from Dr M. P. Bryant, University of Illinois, U.S.A., *Corynebacterium avidum* (1352), *E. aerofaciens* (4109), *E. disciformans* (364B), *E. bifforme* (4213B) from Dr M. Sebald, Pasteur Institute, Paris, *E. ruminantium* (ATCC17233) and *B. rettgeri* (ATCC10825) from the American Type Culture Collection.

The cultures were stained by Gram's method and also to show cell wall structure (*Mackie & McCartney's Handbook of Bacteriology*, 1962). Motility was examined in wet preparations from YE broth and in stab cultures in YE agar containing 0.25% agar. The fermentation of carbon sources using Durham tubes, production of indole, hydrolysis of cellulose and of Oxoid gelatin-charcoal discs and the reduction of nitrate were all tested in a 1% Oxoid peptone base and in a YE broth base.

For the examination of the fermentation products of glucose, the nutrient base (200 ml) was filled into 8 oz medical flat bottles fitted with perforated metal screw caps over rubber liners which allowed samples of gas to be withdrawn using a hypodermic syringe. Glucose was added to 0.5% (w/v) either before sterilization of the base at 115 °C for 10 min, or as a Seitz-filtered solution after sterilization of the base. Either four iron nails or 0.15% cysteine were added aseptically as a reducing agent after sterilization. On one occasion about 4 g iron powder as a reducing agent was sterilized in the medium. The cultures were incubated for 7 days at 37 °C.

The gases in the headspace were analysed by gas-liquid chromatography using argon as the carrier gas (Czerkawski & Clapperton, 1968). The samples for analysis of steam-volatile acids were prepared by the method of Cottyn & Boucque (1968) and the determinations made by gas-liquid chromatography using Carbowax (20 M-TPS on Chromosorb G AW-DMCS, 5 ft × $\frac{1}{8}$ in) (W. G. Pye & Co.). HEF was detected by thin-layer chromatography (Owen & West, 1968). The sulphuric acid-phenol method of Dubois, Gillies, Hamilton, Rebers & Smith (1956) was used to measure the carbohydrate content of the basal media.

RESULTS

The cultures, when grown on RYE agar, initially formed translucent colonies that became bright green due to the production of HEF crystals and, after further incubation, turned crimson. On exposure to the air both green and crimson colonies turned bright orange which contrasted with the more yellow colour of the riboflavin in the medium. These colour changes were assumed to be due to the complex formation of HEF (Miles & Stadtman, 1955). The highly reduced crimson and the oxidized orange colours were the distinguishing features of the isolates. Cultures O and OA were isolated from one rumen sample and 6, 6BO and 6 BOA from another. Cultures ThB and AzA were isolated from the caecum.

Rumen fluid in the media depressed HEF production by bacterial inocula from rumen and caecum. The mixture of acetate, *iso*-butyrate, *iso*-valerate and *n*-valerate reported by Bryant & Robinson (1962) as being necessary for the growth of *Eubacterium* from the rumen of the cow also depressed HEF production by the pure isolates. However, bicarbonate increased HEF production and growth in the presence of the volatile fatty acid mixture of Bryant & Robinson (1962).

Iron, either as a nail or powder, was the reducing agent that gave the best growth. Sodium

Table 1. The characteristics of named and unnamed anaerobic bacterial cultures examined in a YE broth base and a peptone base with an iron nail as reducing agent

	Experimental										B&R*										Bergey									
	O	OA	6	6BO	6BOA	ThB	AzA	No growth in these media																						
Growth at 56 °C	-	-	-	-	-	-	-																							
45 °C	-	-	-	-	-	-	-																							
37 °C	+	+	+	+	+	+	+																							
30 °C	+	+	+	+	+	+	+																							
25 °C	+	+	+	+	+	+	+																							
20 °C	+	+	+	+	+	+	+																							
13 °C	Slow	-	Slow	Slow	Slow	-	Slow																							
Motility	-	-	-	-	-	-	-																							
Indole	-	+	+	+	+	+	1																							
H ₂ S production	-	+	+	+	+	+	+																							
Gelatin hydrolysis	-	-	-	-	-	-	-																							
Cellulose hydrolysis	-	-	-	-	-	-	-																							
HEF production	+	+	+	+	+	+	+																							
Nitrate reduction	-	-	-	-	-	-	-																							
Glucose	AG	AG	AG	AG	AG	AG	AG																							
Maltose	ag	AG	AG	AG	AG	AG	AG																							
Mannitol	- ²	- ²	- ³	- ²	- ²	- ²	- ²																							
Lactose	-	-	-	-	-	-	-																							
Glycerol	- ²	- ³	- ³	- ²	- ²	- ²	- ²																							
Fructose	AG	AG	AG	AG	AG	AG	AG																							
Sucrose	AG	AG	AG	ag	AG	AG	AG																							
Galactose	AG	ag	AG	ag	AG	AG	AG																							
Starch	-	-	-	-	-	-	-																							
Lactate	-	-	-	-	-	-	-																							
Glucose fermentation in YE broth	+	+	+	+	+	+	+																							
Acetic acid	-	-	-	-	-	-	-																							
Propionic acid	-	-	-	-	-	-	-																							
Butyric acid	-	-	-	-	-	-	-																							
CO ₂	+	+	+	+	+	+	+																							
H ₂	+	+	+	+	+	+	+																							

* Bryant & Robinson (1962).

A, a Strong or weak acid production.

G, g Strong or weak gas production.

¹ Positive result.² Negative result.³ Acid and gas in YE broth base.

± Weak reaction.

Table 2. *The percentage of CO₂ and H₂ in the headspace of screw-cap bottles produced by cultures O, 6, AzA from YE broth with and without glucose*

YE broth	Cultures							
	Control		O		6		AzA	
	CO ₂	H ₂	CO ₂	H ₂	CO ₂	H ₂	CO ₂	H ₂
Without glucose	0.12	0.01	1.38	1.36	1.01	0.84	0.93	0.85
+0.5 % glucose	0.03	0.02	49.9	21.9	55.1	31.8	36.5	15.0

dithionite, sodium sulphite and cysteine were tried at various concentrations, but none gave such good growth as reduced iron. In the presence of sodium thioglycolate, growth was very erratic.

In liquid media, the unnamed cultures grew as small lanceolate bacilli 0.3 to $0.5 \mu \times 0.5$ to 1.0μ . On solid media, they tended to form rod-shaped units 3 to 5μ long. A cell-wall stain showed these rod-shaped units to be multicellular. In some cases, chains of rods were formed and this was most noticeable with culture AzA. The bacilli were Gram-positive but were readily decolorized. There was no evidence of spore-formation or branching.

The isolates gave very similar results in all the identification tests and were distinct from the reference cultures (Table 1). None of the isolates grew at 56 or 45°C and if growth occurred at 13°C an incubation time of more than 10 days was required. Optimal growth occurred at 37°C . Motility was not observed in any of the cultures. All cultures produced H_2S from YE broth but strain AzA produced no H_2S from peptone water. The cultures were not strongly odiferous but 2- to 3-week-old cultures had a distinct faecal smell. Indole was not produced. Neither lactate nor nitrate was utilized; gelatin and cellulose were not hydrolysed. The isolates were actively fermentative, producing acid and gas from glucose, maltose, fructose, sucrose and galactose but not from mannitol, lactose, glycerol or starch when either YE broth and peptone water were used as basal media. A small amount of gas was produced from the peptone water and rather more from the YE broth base. The absence of acid was checked using an external indicator dye. When the cultures were grown in screw-cap bottles in YE broth without added glucose, a little gas was formed consisting of equal amounts of CO_2 and H_2 (Table 2) and unidentified carbohydrate material was present in the basal medium equivalent to 0.01% (w/v) glucose in the peptone water and to 0.1% (w/v) in the YE broth. Glucose (0.5%) in the nutrient base gave a marked rise in CO_2 and H_2 production.

After 7 days at 37°C , a small amount of methane was produced in YE broth by cultures 6 and 6 BOA but not by any of the other isolates.

Both with named and unnamed cultures, glucose fermentation was less if glucose was added to the YE broth before sterilization rather than added aseptically as a Seitz-filtered solution (Table 3). Cysteine, as a reducing agent, also depressed glucose fermentation compared to an iron nail in the case of all the cultures examined, except *Butyribacterium rettgeri*. The named and unnamed Eubacterium cultures produced only acetic acid from the fermentation of glucose. *Corynebacterium avidum* produced acetic and propionic acids and *B. rettgeri* produced acetic and butyric acids. Reduced iron powder was not a suitable reducing agent for the examination of fermentation products since it reacted with the acid in the medium to give H_2 gas.

None of the named cultures produced HEF, only the new isolates. Glucose in the medium suppressed HEF production with culture 6 but not with O, ThB or AzA. The production of HEF was a strictly anaerobic reaction and the ability to form this product was less under partial anaerobiosis.

Table 3. The production of CO₂, H₂ (percentage composition of gas produced), acetic (Ac), propionic (Pr) and butyric (But) acids (m-equiv./100 ml YE broth) from the fermentation of 0.5% Seitz-filtered or autoclaved glucose by *Butyribacterium rettgeri*, *Corynebacterium avidum*, *Eubacterium bifforme* and cultures O and 6 with iron nails or cysteine as reducing agent

Glucose reducing agent	Seitz-filtered										Autoclaved with medium									
	Nail					Cysteine					Nail					Cysteine				
	CO ₂	H ₂	Ac	Pr	But	CO ₂	H ₂	Ac	Pr	But	CO ₂	H ₂	Ac	Pr	But	CO ₂	H ₂	Ac	Pr	But
<i>B. rettgeri</i>	40.0	3.3	0.72	.	1.25	29.7	2.6	0.61	.	1.61	27.5	7.4	0.33	0.02	0.46	40.4	17.8	0.41	0.08	1.10
<i>C. avidum</i>	40.2	0.08	1.58	4.70	.	33.2	.	1.02	3.21	.	16.5	0.2	0.97	2.56	.	24.4	0.01	0.80	2.16	.
<i>E. aerofaciens</i>	8.4	2.3	0.27	0.17	.	7.0	0.1	0.094	0.13	.	2.7	0.3	0.23	0.051	.	13.5	.	0.063	0.031	.
<i>E. bifforme</i>	4.4	0.3	0.53	.	.	6.7	0.07	0.37	.	.	Not tested	.	.	.	.	Not tested	.	.	.	.
O	26.9	41.5	1.06	.	.	30.5	54.4	0.73	.	.	32.5	15.2	0.90	.	.	No growth	.	.	.	.
6	34.8	37.1	1.30	.	.	17.2	50.7	0.69	.	.	27.1	17.0	0.88	.	.	No growth	.	.	.	.

DISCUSSION

The isolates described in this work are the first pure cultures to produce HEF from riboflavin. Their morphology, their ability to produce gas from the basal medium, their active fermentation of carbohydrates and the products of glucose fermentation suggested that they were members of the genus *Eubacterium*. Since this genus is not well described in the literature a comparison was made with the species that were reported as showing biochemical reactions similar to those of the new isolates including *Corynebacterium avidum* and *Butyribacterium rettgeri* (*Eubacterium limosum*). The new cultures appeared to be intermediate between the gas-producing species (*E. aerofaciens* and *E. biforme*) and the non-gas-producing species (*E. ruminantium* and *E. disciformans*). They did not ferment lactose, grew more readily than *E. disciformans* in the two basal media used in this work and, unlike *E. ruminantium*, did not require volatile fatty acids for growth.

The production of acid and gas from glucose by the organisms used in this work was influenced to a large extent by the conditions of growth. *Eubacterium aerofaciens*, *E. biforme* and *Corynebacterium avidum* are all reported in the literature as producing acid and gas from glucose. Although this could be confirmed by growth in a closed bottle (Table 3) when the cultures were grown in tubes plugged with cotton-wool and a Durham tube used to collect the gas, *E. aerofaciens* and *E. biforme* produced only acid and no gas, while *C. avidum* did not appear to attack glucose (Table 1). The type of reducing agent and the method used to sterilize the glucose also influenced the products of fermentation (Table 3). The production of gas from the basal medium was presumably due to the fermentation of unidentified carbohydrates in the medium. The buffering capacity of the medium was apparently sufficient to give the appearance of gas formation without the associated acid production that could be detected by an indicator dye. These results show the importance of using a standard method for the growth of this group of anaerobes when fermentation products are to be determined.

The HEF-producing cultures are proposed as a new species: *Eubacterium fissicatena* – a small, easily decolorized Gram-positive lanceolate bacillus 0.3 to $0.5 \mu \times 0.5$ to 1.0μ with a tendency to form chains of multicellular rods 3 to 5μ long; non-motile, non-sporeforming and non-branching; growth range is 13 to 37°C with optimum growth at 37°C ; degrade riboflavin to hydroxyethylflavin.

Old cultures have a faecal odour. They produce H_2S but not indole, they do not utilize nitrate or lactate nor hydrolyse gelatin or cellulose. They produce acid and gas from glucose, maltose, fructose, sucrose and galactose but not from mannitol, lactose, glycerol and starch. The fermentation products of glucose are CO_2 , H_2 and acetic acid. Some strains form methane in the absence of glucose. The GC ratio of strain is 45.4%.

Cultures O, 6 and AzA have been deposited with the National Collection of Industrial Bacteria as *Eubacterium fissicatena* (respectively NCIB10444, 10445 and 10446).

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