

***Peptococcus heliotrinreducans*, sp.nov., a Cytochrome-producing Anaerobe which Metabolizes Pyrrolizidine Alkaloids**

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SUMMARY

Several strains of a new, obligately anaerobic, Gram-positive coccus were isolated from sheep rumen contents. An important distinctive feature was their reductive cleavage of hepatotoxic pyrrolizidines, using hydrogen gas or formate as hydrogen donor. With the same hydrogen donors, the organism reduced nitrate and fumarate. In all cases, the reductive metabolism formed part of an energy-giving sequence used by the organism for growth. This new coccus also utilized energy obtained by dissimilation of arginine. Enzymic hydrolysates of casein and yeast autolysate were satisfactory substrates for growth but no strain fermented carbohydrates. Ultrasonic extracts of the coccus contained a *c*-type cytochrome. The characteristics of the organism are consistent with its allocation to the genus *Peptococcus* within which it differs significantly from currently recognized species. The name *Peptococcus heliotrinreducans* is proposed for this new species, and a reliable method for isolating it is described.

INTRODUCTION

Sheep rumen contents are known to detoxify hepatotoxic pyrrolizidine alkaloids (Dick *et al.*, 1963), a phenomenon of great importance in sheep exposed to pastures containing *Heliotropium europaeum*. The subject has been studied in this laboratory for some years (Lanigan, 1970, 1971, 1972; Lanigan & Smith, 1970), and several strains of a bacterium which brought about reductive cleavage of certain pyrrolizidines have been isolated. These strains resembled one isolated by Russell & Smith (1968), who first demonstrated pyrrolizidine metabolism by a single species, using hydrogen or formate as hydrogen donor and obtaining 7 α -hydroxy-1 α -methyl-8 α -pyrrolizidine and heliotric acid as non-assimilated products from heliotrine. However, the organism described here differs from that of Russell & Smith in certain important characteristics, and it differs significantly from currently recognized species of anaerobic cocci. It is therefore claimed to represent a new species, within the genus *Peptococcus*. The name proposed, *Peptococcus heliotrinreducans*, reflects the important role of this organism in relation to ruminants which ingest plants containing heliotrine and other hepatotoxic pyrrolizidines.

METHODS

Culture methods. Anaerobic culture methods were based on those described by Hungate (1950, 1966, 1969). Oxygen was removed from gases by passing them through heated columns (250 °C) packed with cuprous hydroxide on kieselguhr: the packing had previously been reduced by passing a slow stream of hydrogen through it at the same temperature.

Table 1. *Culture media*

Component	Concentration in medium (g/100 ml)			
	Medium 1	Medium 2	Medium 3	Medium 4 (TYM)
1. Rumen fluid	30.0	30.0	30.0	—
2. Yeast extract (Difco)	0.10	0.10	0.10	1.00
3. K_2HPO_4	0.05	0.05	0.05	0.05
4. KH_2PO_4	0.05	0.05	0.05	0.05
5. NaCl	0.10	0.10	0.10	0.10
6. $(NH_4)_2SO_4$	0.05	0.05	0.05	—
7. $MgSO_4$	0.01	0.01	0.01	0.01
8. $CaCl_2$	0.01	0.01	0.01	0.01
9. $NaHCO_3$	0.60	0.60	0.20	0.60
10. Heliotrine	0.10	0.20	0.10	—
11. Sodium formate	0.05	0.10	—	—
12. Resazurin	0.0001	0.0001	0.0001	0.0001
13. Cysteine-HCl	0.03	0.03	0.03	0.03
14. $Na_2S \cdot 9H_2O$	0.03	0.03	0.03	—
15. Ionagar (Oxoid no. 2)	—	1.00	—	—
16. Tryptone (Oxoid)	—	—	—	1.00
17. Distilled water	Vol. required	Vol. required	Vol. required	Vol. required
18. Gas phase	CO_2	CO_2	$H_2 + CO_2$ (4 + 1)	CO_2

Culture media. Media which provided the basis for development of effective isolation procedures were modelled on those of Paynter & Hungate (1968). The three principal formulations are given in Table 1. In each medium, the pH was adjusted to between 6.7 and 6.8 before sterilization. Rumen fluid, obtained from a fistulated sheep, was strained through six layers of cotton gauze, boiled to coagulate proteins, cooled under nitrogen, and then centrifuged (20000g). The clear supernatant was gassed thoroughly with CO_2 , under which it was stored at 2 to 4 °C. Component 3 (Table 1) was kept as a 0.3 % (w/v) stock solution. A second stock solution contained components 4 to 8 inclusive at six times their concentrations in the medium. To prepare the liquid media, components 1 to 8 and 11 to 12 were combined with the required volume of distilled water, de-aerated by boiling for 1 to 2 min, and cooled with CO_2 bubbling through them. Cysteine hydrochloride and sodium bicarbonate were added when the temperature had fallen below 50 °C. Tubes were filled with the medium under CO_2 , sealed with recessed butyl-rubber stoppers, and held in a press during autoclaving. Aqueous sodium sulphide (1.5 %, w/v) was autoclaved separately under nitrogen and added to the medium just before use.

In preparing medium 2, agar powder was added to each tube before dispensing the solution containing the other components. For medium 3, the solution was protected from oxidation by gassing with CO_2 during preparation, and with an H_2 - CO_2 mixture during dispensing. Tryptone-yeast extract-mineral salts (TYM) medium (medium 4, Table 1) was prepared in a manner analogous to that used for medium 1, and was used for maintaining the organism and for much of the later experimental work.

All other reagents were prepared as solutions of appropriate concentrations, and de-aerated by gassing with CO_2 or N_2 before sterilization. Cysteine hydrochloride for the H_2S production test was sterilized by membrane filtration since it liberated H_2S when autoclaved in TYM medium.

Characterization tests. Methods in *Anaerobe Laboratory Manual* (1973) were followed for the determination of biochemical, physiological and metabolic characteristics of the

organism. Unless otherwise stated, four strains were used for each test. Carbohydrates were tested for growth-stimulating properties in both liquid and solid media. Pathogenicity and toxicity tests were also conducted as recommended by the *Manual*.

Analytical methods. Carbon dioxide, hydrogen, methane and nitrogen were estimated chromatographically using a column of silica gel (80–120 mesh, 4 mm diam. $\times$ 2 m), with argon (15 ml min⁻¹) as carrier. Injection port and column were kept at 50 °C, the thermal conductivity detector at 75 °C, and the bridge current at 150 mA.

Alkaloids and their metabolites were assayed as described by Lanigan & Smith (1970). Preliminary extraction was not necessary since satisfactory separations were obtained by spotting 50 to 70 μ l of culture directly onto the t.l.c. plate.

The guanine plus cytosine (G+C) content of the bacterial DNA was calculated from buoyant-density values (Schildkraut, Marmur & Doty, 1962). Cells were washed, re-suspended in saline-EDTA (Marmur, 1961) at an extinction of 1.8 to 2.0 at 600 nm, and the suspension was passed three times through a French press and centrifuged at 20000g to remove cells and debris. The supernatant was used without further purification for DNA buoyant-density determination in caesium chloride using a Beckman Model E analytical centrifuge fitted with a monochromator and photoelectric scanning optics. The scanner electronics were interfaced to a mini-computer (Data General Nova). Operating conditions were 25 °C and 44000 rev./min for 68 to 72 h. The average signal from ten consecutive scans of the ultracentrifuge cell, at 265 nm, was used to calculate the buoyant density.

RESULTS

Isolation of the organism

The strains studied were isolated from pen-fed Merino $\times$ Border Leicester ewes and wethers. Inclusion of dried *Heliotropium europaeum* in the ration favoured growth of the organism in the rumen, but was not a prerequisite. Enrichment and dilution culture procedures, used initially, were rarely successful. But the isolation of an active strain and subsequent recognition of the competition between it and methanogenic species for metabolic hydrogen (Lanigan, 1971) made possible the development of the following procedure for consistent isolation of the heliotrine-metabolizing coccus.

Sheep rumen contents were strained through gauze. Heliotrine (2 mg ml⁻¹) and chloral hydrate (0.2 mg ml⁻¹) were added, and the flask was gassed with H₂-CO₂ mixture (4:1) before sealing with a rubber stopper. This primary enrichment culture was incubated at 39 °C with constant shaking, until almost all the heliotrine had been metabolized (24 to 30 h). Successive subcultures were then made in medium 3, until, with 0.5 % inoculum, the time for utilization of the heliotrine became minimal (about 16 h). Such a culture was serially diluted in medium 2 and anaerobic roll tubes prepared. After 7 to 8 days at 38 °C, colonies of the type formed by the organism sought were picked out into medium 1, incubated for 3 days and tested chromatographically for heliotrine breakdown. Active cultures were re-passaged through roll tubes until purity was established. Stock cultures held in roll tubes at 2 to 4 °C remained viable for at least a year. Working stocks were maintained by two-weekly transfer (3 days at 38 °C and 11 days at room temperature), initially in medium 1, but, more recently, in TYM medium.

Nine strains, designated RHS 1 to 9, were isolated from sheep maintained on various dry rations. An identical organism was recovered from a rumen-fistulated sheep fed on lucerne pellets, at the University of California in Davis, U.S.A. Attempts to isolate the organism from cattle rumen contents met with only limited success: crude active enrichment cultures

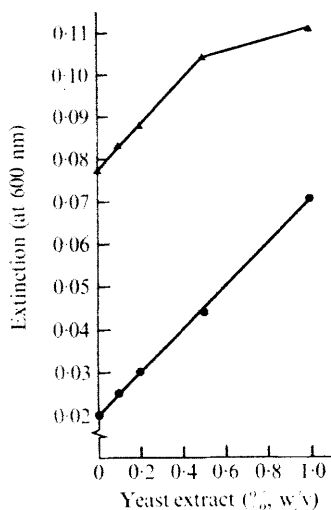


Fig. 1

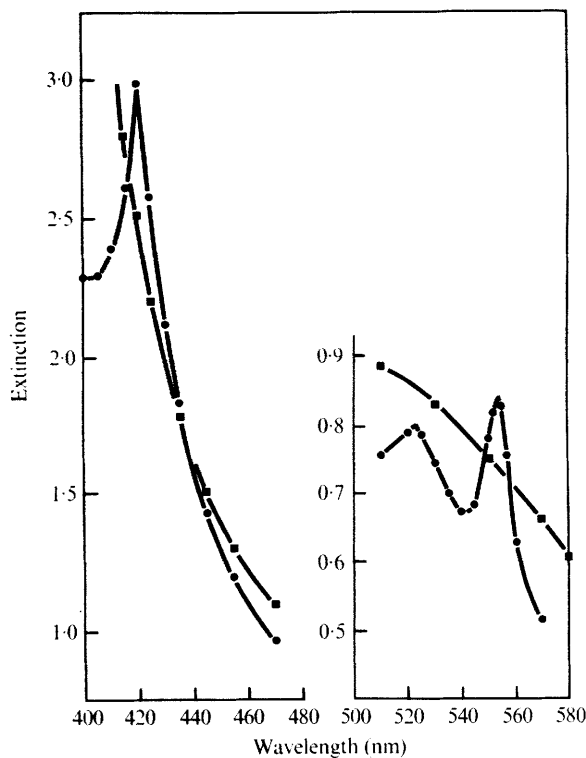


Fig. 2

Fig. 1. Growth of strain RHS 1 in TYM medium with different concentrations of tryptone (Oxoid) and yeast extract (Difco): ●, 0.5 % tryptone; ▲, 2.0 % tryptone.

Fig. 2. Absorption spectra of cell-free extracts of strain RHS 4. ■, Oxidized, extracts prepared under CO_2 ; ●, reduced, dithionite added to contents of cuvette. Cells were grown in nitrate-formate medium.

were readily obtained but only two isolates displayed a very limited capacity for heliotrine metabolism in pure culture. No further work was done with these strains.

Nutrition and physiology of the organism

All strains were strict anaerobes, and soon died in media in which the resazurin indicator became oxidized. On first isolation, the reducing combination of cysteine plus sodium sulphide appeared to be essential, but, after maintenance in the laboratory for over a year, all strains grew with cysteine alone. No attempt was made to determine precisely the organism's tolerance for oxygen.

An enzymic digest of casein (Oxoid tryptone) or yeast autolysate (Difco) supplemented with mineral salts was an adequate substrate for growth. With limiting concentrations of one of these substrates, growth response to increasing concentrations of the other was linear from zero to a combined concentration of 2 to 2.5 g/100 ml. There was no evidence from the shape of the curves to indicate that yeast extract, in particular, served as a source of an essential accessory growth factor (Fig. 1).

Several pyrrolizidines, in addition to heliotrine, could be used by the organism for oxidation of formate and hydrogen. Thus europine, heleurine, supinine and lasiocarpine

Table 2. *Effect of different hydrogen acceptors on growth in formate-rumen fluid media*

All three hydrogen acceptors were used at 3 mM concentration, and formate was present in stoichiometric excess.

Hydrogen acceptor	<i>E</i> _{600 nm} at maximal growth		
	Strain RHS1	Strain RHS2	Strain RHS4
Heliotrine	0.080	0.070	0.080
Fumarate	0.072	0.053	0.067
Nitrate	0.282	0.246	0.258
None	0.028	0.023	0.030

Table 3. *Effect of rumen fluid on heliotrine metabolism and growth on nitrate, in the presence of excess formate*

	Heliotrine metabolized (mg/ml)		Growth on nitrate (max. <i>E</i> _{600 nm})	
	Strain RHS1	Strain RHS4	Strain RHS1	Strain RHS4
TYM*	0.5	0.5	0.070	0.065
TYM+rumen fluid*	> 2.0	> 2.0	0.225	0.160

* Tryptone, 1.0 g/100 ml; yeast extract, 0.2 g/100 ml; rumen fluid, 30 % (v/v).

were converted to 1-methylene derivatives and supported growth in medium 1. None of these alkaloids was converted to a 1-methyl derivative by the organism although this type of compound was found when they were incubated with rumen contents. Some macrocyclic di-ester pyrrolizidines, e.g. crispatine and monocrotaline, were metabolized, but more slowly than were the mono-esters. Metabolism of these two compounds was increased when heliotrine (0.5 mg ml⁻¹) was also present in the medium. Heliotridine, anacrotine, retrorsine and the saturated pyrrolizidines, cynaustaline and sarracine, were not metabolized, even in the presence of heliotrine.

In addition to the pyrrolizidines, the organism utilized fumarate and nitrate as hydrogen acceptors for formate oxidation. In medium 1, with excess formate and the hydrogen acceptors in equimolar concentrations, approximately equal growth was obtained with heliotrine and fumarate but with nitrate, growth was at least 3.5 times greater. Nitrite was detected after 24 h in nitrate media when hydrogen donors were limiting, e.g. in TYM medium with 0.1 % potassium nitrate. Table 2 compares the growth of three strains in medium 1 with either heliotrine, fumarate or nitrate as hydrogen acceptor.

The carbohydrates, glucose, fructose, galactose, mannose, cellobiose, xylose, lactose, maltose, sucrose, melibiose and starch were not fermented by the organism, and they did not stimulate the growth of any strain.

The organism liberated ammonia from tryptone, yeast extract, nitrates (with formate present), adenine, uracil and arginine. It was of particular interest that dissimilation of arginine resulted in a marked stimulation of growth in TYM medium, the extent of which varied from strain to strain as did the arginine concentration producing maximal effect (10 to 25 mM). Growth was not stimulated by the addition of any of several other amino acids to TYM media where the total of tryptone and yeast extract was growth limiting. Thus alanine, glutamate, histidine, ornithine, proline, serine, threonine and the

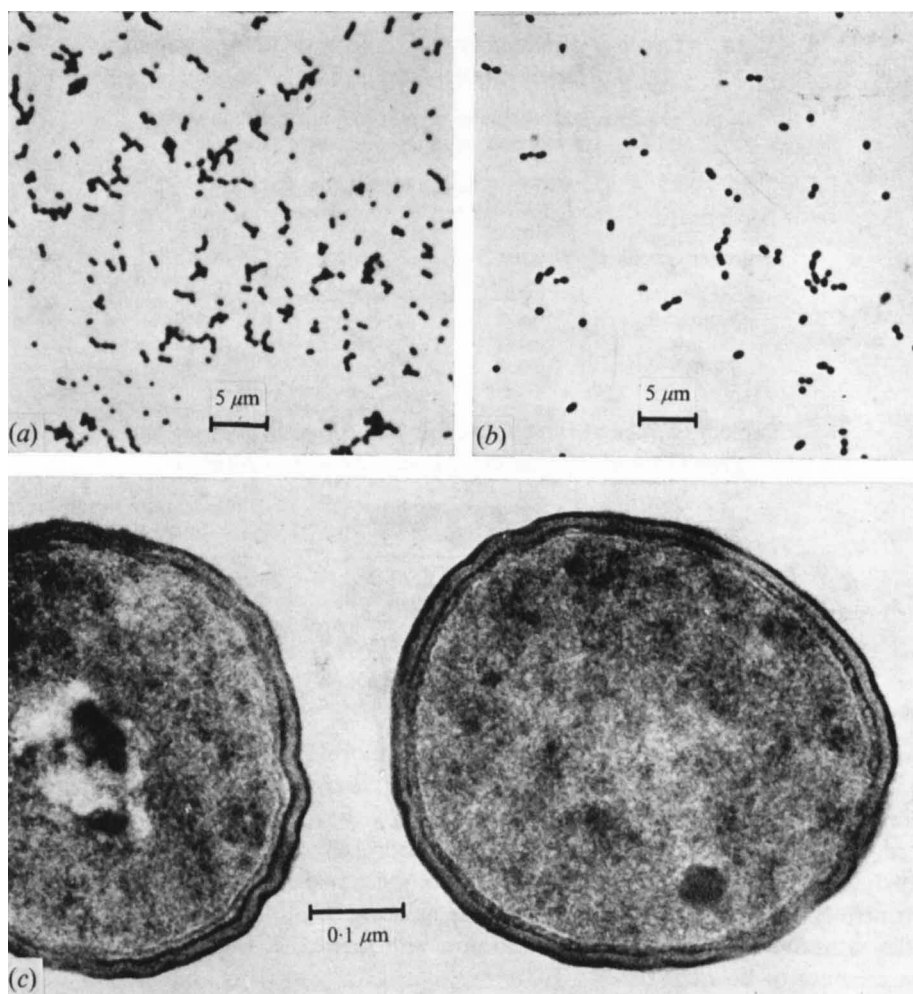


Fig. 3. Morphological features of strain RHS 1. (a) Gram-stained smear of cells grown in nitrate-formate medium. (b) Phase-contrast preparation of cells grown in heliotrine-formate medium. (c) Electron micrograph of thin sections of cells grown in nitrate-formate medium.

combinations, alanine plus ornithine and alanine plus proline, did not affect growth. Glycine (40 mM) was inhibitory to all strains.

Rumen fluid was not essential for growth in TYM medium, but if the concentration of yeast extract was decreased below 0.2 g/100 ml, addition of 30 % (v/v) rumen fluid did produce a small increase in growth. In contrast to this limited effect, the same proportion of rumen fluid greatly enhanced heliotrine metabolism and growth on formate plus nitrate (Table 3).

The cell pellet in media containing nitrate and a stoichiometric excess of formate was distinctly pink in colour. Therefore the possible presence of cytochromes was investigated. Ultrasonic extracts of strains RHS1 and 4, prepared under CO_2 , showed only one absorption peak, at 410 nm, within the range 400 to 600 nm but when a crystal of sodium dithionite was added to the cuvette, the Soret peak shifted to 420 nm and two new peaks appeared, one at between 522 and 523 nm and the other at 553 nm. Later, similar results were obtained

Table 4. Relationship of the pyrrolizidine-metabolizing coccus to the genera of Peptococcaceae

Data for genera are taken from Rogosa (1971), *Bergey's Manual* (1974) and Holdeman & Moore (1974). Where + and - are given, the first represents the reaction of the majority of species.

	Cell arrangement		Growth at pH 2.5	G + C (mol %)	Carbo- hydrates fer- mented	Cellu- lose fer- mented	Used peptones or amino acids as main N and energy source	Nitrates reduced
	Chains	Cubic packets						
<i>Peptococcus</i>	Not characteristic	-	-	35.7-36.7	- or +	-	+	+ or -
<i>Peptostreptococcus</i>	Characteristic	-	-	33.5	+ or -	-	+	-
<i>Ruminococcus</i>	+ or -	-	-	39.8-45.4	+	+	-	-
<i>Coprococcus</i>	+ or -	-	NR	39-42	+	-	-	-
<i>Sarcina</i>	-	+	+	28.6-30.6	+	-	-	NR
Pyrrolizidine- metabolizing coccus	Not characteristic	-	-	35.4-36.9*	-	-	+	+

* Range of values for six strains, buoyant-density method. NR = not recorded.

with heliotrine-grown cells. These findings indicated the presence of a *c*-type cytochrome. No shoulders were present on the 522 or 553 nm peaks, thus indicating the absence of *b*-cytochromes. Strain 4 absorption spectra are shown in Fig. 2.

Taxonomic relationships

Major properties of the new coccus are shown with those of the genera of Peptococcaceae in Table 4. Clearly, the organism is a species of *Peptococcus*. Table 5 compares characteristics of the pyrrolizidine-metabolizing *Peptococcus* with those of currently accepted species of this genus.

Description of the organism

Proposed name. *Peptococcus heliotrinreducans*, sp.nov., derived from heliotrine, a pyrrolizidine alkaloid, and Latin adj. *reducans* = reducing, referring to the organism's ability to bring about reductive cleavage of the heliotrine molecule.

Cell and colony morphology. Obligately anaerobic, non-motile, non-sporing, spherical to slightly elongated cocci, that occur singly, in pairs, in small clusters and occasionally in short chains of 3 to 6 cells. Cells are Gram-positive, 0.5 to 0.6 μ m diam. in stained smears, but up to 0.7 μ m in wet preparations. No capsules are formed (Fig. 3*a*, *b* and *c*).

Deep colonies in anaerobic tryptone-yeast-mineral salts agar are lenticular with an entire margin and smooth surface, greyish-white, translucent, and 0.6 to 0.8 mm diam. after 7 to 10 days. Surface colonies are 1.5 to 2.5 times the diameter of deep colonies, effuse, with an entire edge, virtually colourless and transparent. A few large colonies display varying amounts of radial striation and crenation of the edge. Streak growth is beaded with a slightly raised confluent margin, glistening, colourless and transparent.

General cultural and physiological characteristics. In liquid TYM medium, after a slight initial turbidity, the cells form a highly viscous pellet which is difficult to disperse. Growth is maximal in 24 to 30 h. In the absence of bicarbonate, and using gaseous nitrogen, growth

Table 5. *Comparison of the pyrrolizidine-metabolizing coccus with currently accepted species of Peptococcus*

Data are taken from *Bergeys' Manual* (1974).

	<i>P. niger</i>	<i>P. asaccharolyticus</i>	<i>P. aerogenes</i>	<i>P. activus</i>	<i>P. constellationis</i>	<i>P. anaerobius</i>	New Peptococcus
Sugars fermented	—	—	W	+	+	—W	—
Visible gas in non-sugar media	+	+	+	+	—	—	—
Glycine fermented	NR	—	—	—	NR	+	—
Serine, threonine, glutamate and histidine fermented	NR	+	+	+	NR	—	—
Purines fermented	NR	+	+	+	NR	—	+
Nitrates reduced	NR	+	+	+	NR	—	+
Indole	NR	+	+	+	—	—	—
Gelatine liquefied	—	—	—	+	—	—	—
H ₂ S	+	+	+	+	NR	+	—

NR = not recorded, + = positive result, — = negative result, W = feebly positive.

* Adenine was the only purine tested.

is reduced by up to 50 %. Either tryptone or yeast extract alone is an adequate substrate for growth. Rumen fluid does not stimulate growth in TYM medium. No visible gas is formed in heavily inoculated deep tubes of TYM agar.

The temperature optimum for growth in TYM medium is 40 °C. All strains grow within the range 30 to 46 °C and maximally between 38 and 42 °C. The organism does not survive at 60 °C for 30 min. All strains give maximal growth between pH 6.5 and 7.0, and more than 90% of the maximum growth at pH 6.2 and 7.2. No growth occurs below pH 5.4 or above pH 8.2. Salt tolerance is relatively low: growth is markedly decreased in TYM containing 1 % NaCl; and there is no growth with 2 % NaCl. Growth is not appreciably affected by addition of serum (1 %) or Tween 80 (1 %).

Cooked meat and litmus milk media, supplemented with cysteine, are unchanged although some growth occurs. No growth occurs in blood agar. Penicillin G (10 units/ml) completely inhibits growth in TYM.

Biochemical and metabolic properties. Carbohydrates are not fermented, neither do they stimulate the growth of any strain. Starch is not hydrolysed. Lactate, malate and pyruvate are not fermented. H₂S is not produced from cysteine. Sulphates are not reduced in the presence of formate or hydrogen. Indole is not formed in TYM medium. Gelatin is not liquefied. Urea is not hydrolysed. No catalase is formed.

Heliotrine and several other pyrrolizidine alkaloids with a C₁-C₂ double bond, are converted to 1-methylene derivatives, with hydrogen or formate serving as hydrogen-donor. Nitrates are reduced to nitrites and, in the presence of sufficient hydrogen or formate, are further reduced to ammonia. Both nitrate and heliotrine metabolism are stimulated by addition of rumen fluid (30 %, v/v) to the medium. Fumarate is reduced to succinate in the presence of hydrogen or formate. A *c*-type cytochrome is present in cells grown in heliotrine and nitrate media.

Ammonia is produced in TYM medium and in substantially increased concentration when arginine is added. Growth of the organism is markedly increased by arginine. Several other amino acids do not stimulate growth. Glycine (40 mM) is inhibitory. Creatinine is not hydrolysed. Purines and pyrimidines are fermented (1 to 2 mM-adenine and 3 to 5 mM-uracil).

Metabolic products in TYM are acetic, propionic and butyric acids in low concentrations, with a trace of isovaleric acid. The concentration of isovaleric acid is increased when arginine is added. Lactate and succinate are not formed in TYM, but, in the presence of hydrogen or formate, succinate is formed from fumarate. Ethanol is not detectable.

Hydrogen is liberated in small amounts from rumen fluid-yeast extract media containing a bicarbonate buffer. Carbon dioxide is liberated in small amounts from TYM when bicarbonate is omitted and nitrogen is substituted for carbon dioxide in the initial gas phase.

The G+C content of the DNA is 36.1 (35.4 to 36.9 for six strains) mol % (buoyant-density method).

Cells are non-pathogenic for guinea pigs (intramuscularly); and non-toxic for mice (culture filtrate intraperitoneally).

Source: isolated from the rumen contents of sheep.

Type strain: RHS1. Cultures of the type strain have been deposited with the NCTC, London, (no. 11029) and the ATCC, Washington, D.C., U.S.A., (no. 29202).

DISCUSSION

The family Peptococcaceae (*Bergey's Manual of Determinative Bacteriology*, 1974) comprises four genera of Gram-positive, anaerobic, chemo-organotrophic cocci with complex nutritional requirements: *Peptococcus*, *Peptostreptococcus*, *Ruminococcus* and *Sarcina*. More recently, Holdeman & Moore (1974) described a new genus, *Coprococcus*, which they claimed should be included in Peptococcaceae. The rumen coccus described in this paper is clearly not a member of *Ruminococcus*, *Sarcina* or *Coprococcus*; it is not a carbohydrate fermenter; it uses protein hydrolysates as its main nitrogen and energy sources; and the guanine plus cytosine content of its DNA is distinctly different. The distinctions between *Peptococcus* and *Peptostreptococcus* are not, in the author's view, clear-cut (Rogosa, 1971) and a good case could be made for combining the two genera until a more definite basis for their separation is established. However, according to present definitions of the genera, the new rumen coccus is best allocated to *Peptococcus* on the basis of its G+C ratio, non-fermentation of carbohydrates, assimilatory reduction of nitrates and morphology. Within the genus *Peptococcus*, *P. aerogenes*, *P. activus* and *P. constellatus* differ from the new strains in that they ferment sugars and the amino acids serine, threonine, glutamate and histidine, produce gas in non-carbohydrate media, and form H₂S and indole. Carbohydrate fermentation is a doubtful property of *P. aerogenes* but its fermentation of glycine, H₂S production, non-fermentation of adenine, and inability to reduce nitrates provide a clear distinction between this species and the new organism. The latter is also readily distinguished from *P. niger* and *P. asaccharolyticus* with regard to gas production, amino-acid fermentation and indole and H₂S production (see Table 5). The rumen coccus isolated by Russell & Smith (1968), while causing an identical cleavage of heliotrine, differed from the strains described here in certain important properties: it was Gram-negative, used at least 14 carbohydrates for growth, failed to grow if bicarbonate plus CO₂ were replaced by phosphate plus nitrogen and could not grow at pH values greater than 7.0. While the identity of the earlier isolate is not clear, it seems that more than one bacterial species is involved in rumen pyrrolizidine metabolism. The name chosen for the proposed new species, *Peptococcus heliotrinreducans*, is intended to highlight an important role of this organism in nature. It is recognized, however, that the capacity for reductive cleavage of pyrrolizidines cannot have taxonomic significance until other species have been tested for it.

The mechanisms by which *P. heliotrinreducans* can use such dissimilar compounds as fumarate, nitrate and pyrrolizidines as hydrogen acceptors for the oxidation of formate

and hydrogen, thereby gaining useful energy for growth, remain to be elucidated. This bacterium joins a growing list of strict anaerobes which form cytochromes. Jacobs & Wolin (1963) produced evidence that *Vibrio succinogenes* (Wolin, Wolin & Jacobs, 1961) used *b*-cytochromes in electron transport related to formate and hydrogen oxidation, while Macy, Probst and Gottschalk (1975) concluded that cytochromes were involved in fumarate reduction by the strict anaerobe, *Bacteroides fragilis*. Similar relationships may well exist in *P. heliotrinreducans* and their investigation could prove a fruitful area for future research.

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