***Streptococcus pleomorphus* sp.nov.: An Anaerobic Streptococcus Isolated Mainly from the Caeca of Birds**

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A new species *Streptococcus pleomorphus* is described. It is obligately anaerobic and classified in the genus *Streptococcus* because it is a Gram-positive coccus growing in pairs and chains, with a homolactic fermentation of glucose producing L-lactic acid. The GC content of the DNA is 39 mol %. The organism has been mainly isolated from chickens, turkeys and ducks.

INTRODUCTION

Until recently, Gram-positive anaerobic cocci which occurred in pairs and chains were included in the genus *Peptostreptococcus*, classified in the seventh edition of *Bergey's Manual of Determinative Bacteriology* (Breed, Murray & Smith, 1957) within the family Lactobacillaceae. Rogosa (1971) established a new family Peptococcaceae, containing the genera *Peptococcus*, *Ruminococcus* and *Peptostreptococcus*. This family was defined as containing cocci which did not produce lactic acid as a major fermentation product. Later, in discussing the genus *Peptostreptococcus*, Rogosa (1974) suggested that organisms which produced lactic acid as a major fermentation product should be included within the genus *Streptococcus*, a view supported by Holdeman & Moore (1974). Amongst the anaerobic cocci occurring in pairs and chains which have been isolated by us from poultry, a number have now been shown to produce lactic acid as the major fermentation product. These include groups 1 and 3 peptostreptococci described by Barnes & Impey (1970) and the uric acid decomposing strain of Barnes & Impey (1974).

The group 1 strains of Barnes & Impey (1970) are identical with the anaerobic coccus group VIa strains of Hare (1967), and further tests in our laboratory (unpublished data) have confirmed that they have a homolactic fermentation and are aerotolerant, growing equally well in air containing 10% CO₂, hydrogen or hydrogen containing 10% CO₂. In our opinion these bacteria are members of the species *Streptococcus intermedius*, as defined by Holdeman & Moore (1974).

In view of the reappraisal of the genus *Peptostreptococcus* (Rogosa, 1974), we have examined strains of group 3 peptostreptococci (Barnes & Impey, 1970) in an attempt to clarify their taxonomic status. To this end, particular attention was paid to those properties which would justify their inclusion in the genus *Streptococcus* either as a new species or as one of the aerotolerant or anaerobic species redefined or described by Holdeman & Moore (1974). At the same time they have been compared with the only existing strain of the anaerobic coccus group VIIa of Hare (1967). The results are presented here.

METHODS

Origin of strains. Nine strains, all identified as group 3 peptostreptococci (Barnes & Impey, 1970), were isolated in these laboratories in a number of different experiments. Strains EBF61/31B, EBF61/60B, EBF61/66, EBF61/67, EBF61/68 and EBF61/69 were from the caeca of 12-week-old chickens, strain EBD1/3B from the caeca of a duck and strains EBT2/61 and EBT2/68 from the vent area of a frozen turkey carcass. Strain NCTC9808, anaerobic coccus group VIIa, was described by Hare (1967).

Strains used for comparison were the group 1 *Peptostreptococcus* strain EBF77/25 (Barnes & Impey, 1970) and the anaerobic coccus group VIa strain NCTC9817 (Hare, 1967), both now identified as *Streptococcus intermedius* (Holdeman & Moore, 1974).

The cultures were preserved by freeze-drying (Barnes & Goldberg, 1962).

Morphological and physiological tests. The majority of tests were carried out using the anaerobic techniques, media and methods described by Barnes & Impey (1968, 1970). In order to make valid comparisons with those anaerobes, particularly other cocci, which will only grow when strict precautions are taken to eliminate all traces of oxygen from the environment, the primary characterization of the strains was also carried out using the technique of Hungate (1950). Tests included morphological studies, the utilization of certain carbon sources for growth and the analyses for fermentation products. The media and methods used were those described by Barnes & Impey (1974).

Catalase. The organisms were grown on VL and BGPhf agar. After 3 days incubation the anaerobic plates were exposed to air for at least 1 h and any catalase activity was detected by the formation of gas bubbles after the addition of 3% (v/v) H_2O_2 to the colony.

All incubations were at 37 °C except when otherwise indicated.

Media. The growth media used for the morphological studies or for temporary maintenance of these organisms were as follows: SM10 (Barnes & Impey, 1974) used with the Hungate technique; VL, modified from Beerens & Tahon-Castel (1965) by Barnes & Impey (1968); VL blood agar consisting of VL agar overpoured with a layer of VL agar containing 5% (v/v) horse blood; BGP (Barnes & Impey, 1968) used alone, or supplemented with haemin, liver and chicken faecal extract (BGPhf) (Barnes & Impey, 1970); and RCM (Hirsch & Grinstead, 1954).

Serological methods. Antisera of *Streptococcus* groups A–H, K–S were from Wellcome Research Laboratories. Precipitin tests were carried out as described by Medrek & Barnes (1962). The organisms were grown in basal medium (Barnes & Impey, 1968) without agar and containing 1% (w/v) glucose. Before inoculation the medium was held in a boiling water bath for 20 min to remove oxygen, and the tubes were incubated in an anaerobic jar under hydrogen containing 10% CO_2 for 2 days at 37 °C.

Examination of the products of glucose fermentation

Growth of the organism. Bacteria were grown in a 500 ml Florence flask containing 200 ml of the following medium (g l⁻¹): tryptone (Oxoid), 7.5; beef extract (Lab-Lemco, Oxoid), 4; yeast extract (Oxoid), 3; cysteine hydrochloride, 0.5; glucose, 5; KH_2PO_4 , 4; pH 7.2 to 7.4. The medium was sterilized by autoclaving at 121 °C for 15 min, cooled rapidly to 37 °C and 1 ml of 0.5 M-NaHCO₃, (sterilized by autoclaving) was added followed by an inoculum of 4 ml of 16 h culture grown in VL medium. The flask was placed at 37 °C and a gassing head was fitted with the outlet connected to a trap containing 20 ml of carbonate-free 2 M-NaOH to absorb any CO_2 produced during the fermentation. A tube of soda lime, at the exit from the trap, prevented ingress of atmospheric CO_2 . Immediately after inoculation and assembly, hydrogen was bubbled through the medium for 20 min at approximately 200 ml min⁻¹; the inlet to the gassing head was then closed.

After incubation for 24 h, 2 ml of 5 M-H₂SO₄ contained in a tube suspended inside the flask was tipped into the culture to release any combined CO_2 which was then flushed through the absorber by a slow stream of oxygen-free nitrogen for 1 h. The flask was then disconnected, the volume of liquid was measured and the bacteria were recovered by centrifuging at 15000 g for 20 min. The supernatant was kept at -20 °C until analysed. The bacteria were washed with distilled water, re-centrifuged and suspended in 5 ml distilled water; samples (1 ml) of this suspension were dried at 110 °C for 24 h to determine the dry weight.

Analysis of culture supernatants. (i) Glucose was estimated with glucose oxidase following the manufacturer's instructions (Boehringer GOD-Perid method).

(ii) Carbon dioxide absorbed by the NaOH in the trap was estimated manometrically by liberation with acid. A sample of the original 2 M-NaOH solution which had been kept stoppered throughout the incubation and a sample from the uninoculated medium were used as controls.

(iii) Volatile fatty acids were separated from the fermentation supernatant by the steam distillation method of Annison (1954) but with a distillation rate of approximately 500 ml h⁻¹. Two 40 ml portions of distillate were collected and each titrated under CO_2 -free conditions using 0.1 M-NaOH from a microburette

(Manostat Corporation, New York, U.S.A.) and phenol red indicator. After titration, a further 0.2 ml of 0.1 M-NaOH was added to each portion; these were then combined and evaporated to dryness. Before separation of the individual acids, 0.1 ml of *n*-hexanoic acid [2 % (w/v) in an aqueous solution of acetone (40 %, v/v)] was added as an internal standard to the dried sodium salts. Approximately 20 % excess of 0.75 M-H₃PO₄ was then added together with distilled water to bring the final volume to 0.4 ml. For quantitative separation of the volatile acids other than formate, 1 µl samples were analysed by gas chromatography using a dual flame ionization detector and a glass column (1.5 m × 4 mm) packed with polyethylene glycol 20M (20 %, w/v) on 100/120 mesh Diatomite C, washed with H₃PO₄ (2 %, w/v). The column temperature was 145 °C, detector temperature 200 °C and the carrier gas was argon flowing at 50 ml min⁻¹. The concentrations of individual acids, except formic acid to which the detectors were insensitive, were determined from the ratios of their peak areas to that of the internal standard, corrected by previously determined detector response factors. For the determination of formate, the following reagents were added to the dried sodium salts: 0.1 ml of 1 % (w/v) tetradecane in acetone, as an internal standard; 50 % excess of 0.5 M-dichloroacetic acid in acetone (Shelley, Salwin & Horwitz, 1963); and acetone to bring the total volume to 0.5 ml. Samples (1 µl) were analysed by gas chromatography using a ⁹⁰Sr-argon ionization detector and a glass column (1.3 m × 4 mm) with the packing described above. The column temperature was 100 °C and the flow rate of argon carrier gas was 50 ml min⁻¹.

(iv) Total lactic acid was estimated by the ceric sulphate method of Elsden & Gibson (1954).

(v) L-Lactic acid was estimated by the method of Grey, Jones & Robinson (1974) using L-lactate dehydrogenase (cytochrome b₂).

(vi) Succinic acid in culture supernatants (20 ml) was extracted with celite and ether by the method of Knight (1962) for the recovery of lactate during malate degradation. After evaporation of the ether, the residue was dissolved in 0.4 ml water and succinic acid was estimated polarographically in the apparatus described by Peel (1963). The reaction chamber, at 37 °C, contained 2 ml of 0.1 M-phosphate buffer pH 7.4, 1 ml crude succinic oxidase from pig heart (Umbreit, Burris & Stauffer, 1964), and 3 ml distilled water. After equilibration, 0.04 ml of dissolved residue was added and the oxygen uptake was compared with that caused by the addition of the same volume of 0.02 M-succinic acid.

DNA base composition. Cultures were grown as described for the glucose fermentation experiments except that the medium and inoculum were increased sixfold and the gas phase was nitrogen. Growth was measured turbidimetrically on samples withdrawn through a port in the gassing head, under a flow of nitrogen. Cultures in the late-exponential phase of growth (24 h) were harvested by centrifugation and washed twice with saline/EDTA solution (containing 0.15 M-NaCl and 0.1 M-EDTA, pH 8.0). Bacteria were disrupted in a press (Hughes, 1951) and then heated at 70 °C for 15 min in saline/EDTA containing 3 % (w/v) sodium dodecyl sulphate. The method of Marmur (1961) was then used with the following modifications. Three ribonuclease treatments were given at 37 °C for 0.5 h, using ribonuclease T1 in addition to ribonuclease I (Saito & Miura, 1963). After each treatment, NaCl and heat-treated protease (Sigma type VI) (Hotta & Bassel, 1965) were added for 1 h at 45 °C (1 mg protease preparation per ml nucleic acid solution for the first treatments; 100 µg ml⁻¹ for the second and third treatments). After the first two protease treatments a single deproteinization was done but after the third treatment the procedure of Marmur was followed, with further deproteinization and one final precipitation using propan-2-ol. The precipitated DNA was dried with ethanol and dissolved in 0.15 M-NaCl plus 0.015 M-trisodium citrate.

To determine T_m , samples of this solution with an initial $E_{260}^{1\text{cm}}$ of approximately 0.5, were heated electrically in semi-micro cuvettes from 77 to 97 °C at 0.2 °C min⁻¹. The temperature was measured by a calibrated thermocouple sealed into the Teflon stopper and positioned just above the light path. Values of transmittance and thermocouple e.m.f. were logged every 20 s on a punched paper tape by a Solartron Data Transfer Unit having a digital voltmeter type LM 1604 and a Facit 4070 paper tape punch. Absorbance relative to that at 25 °C and temperature in °C were obtained by a Fortran program run on an IBM 1130 computer. These values were plotted by an IBM 1627 plotter to produce a denaturation curve from which the T_m was measured and the base ratio (as mol % GC) was calculated according to the equation: mol % GC = $(T_m - 69.37)/0.41$ (De Ley, 1970). DNA from *Escherichia coli* B (Sigma type VIII) was used as a standard and gave a value of 90.9 ± 0.3 °C [mean of 11 determinations compared with published values of 89.7 to 91.5 °C (Colwell & Mandel, 1964; Hoyer & McCullough, 1968; De Ley, 1970)].

RESULTS

The group 3 peptostreptococci (Barnes & Impey, 1970) studied in this paper occurred in the avian caeca at $> 10^7 \text{ g}^{-1}$. They were obligate anaerobes, being unable to grow on an optimal growth medium (BGPhlf agar) when incubated in air or air containing 10% CO_2 , but they could be isolated under anaerobic conditions which would not permit growth of the more fastidious anaerobes which considerably outnumbered them in the caecum. Selective media have not been developed for their isolation but some strains, including EBF61/60B, were recovered on the ethyl violet azide medium developed by Barnes & Goldberg (1962) for the isolation of *Bacteroides hypermegas*. Subsequent tests showed that they could not be recovered quantitatively and growth of some strains was inhibited on this medium. Other strains were isolated on VL blood agar containing 100 μg kanamycin sulphate ml^{-1} (Bayer Products Co., Surbiton-upon-Thames, Surrey).

Morphology and cultural characteristics

The nine strains of group 3 peptostreptococci and the anaerobic coccus group VIIa strain (NCTC9808) were all characterized by extreme pleomorphism and variations in cell size. Typical cocci in pairs and chains occurred in some media whilst in other media they were always coccobacillary. Whether this pleomorphism depends on media constituents or the anaerobic conditions used has not been established. An example of the differences obtained can be seen in Fig. 1 where the same 24 h culture of EBF61/60B was used to inoculate VL broth, subsequently incubated for 24 h, SM10 broth, incubated for 24 h (using the Hungate technique), and a BGPhlf agar plate, incubated under hydrogen containing 10% CO_2 for 3 days. No capsules were observed. The organisms were Gram-positive in very young cultures, often becoming Gram-negative within 24 h.

All strains grew well in RCM, VL or BGP liquid media without supplements (see Methods). After 3 days incubation, colonies on agar media such as RCM, BGPhlf, VL or VL blood agar were about 2 to 3 mm in diameter, had an irregular edge and were cream. On the VL blood agar, β -haemolysis was sometimes noted but appeared to be a variable character.

Serology

The group 3 *Peptostreptococcus* strain EBF61/60B and the anaerobic coccus group VIIa strain (NCTC9808) were tested serologically (see Methods) and found to have no reaction with antisera of *Streptococcus* groups A-H, K-S; there was a slight reaction with group O antiserum.

Physiological properties

The physiological properties are listed in Table 1 and in the description of the species given below.

Although carbohydrates were required for growth only a few were utilized, in particular glucose, fructose and usually mannose. Tests carried out by Barnes & Impey (1972) using strain EBF61/60B and substrates of possible ecological importance in the avian caecum, showed no activity against dextran, xylan, starch, amorphous cellulose, tributyrin, casein, chondroitin sulphate, uric acid, deoxyribonucleic and ribonucleic acids; but weak pectinolytic activity was observed.

Glucose metabolism

A detailed study of the glucose metabolism of strain EBF61/60B showed that the main fermentation product was L-lactic acid, formed in sufficient amount to account for all the glucose utilized (Table 2). Carbon dioxide, formic, acetic, *n*-butyric and succinic acids were produced but in much smaller amounts and may well have been derived from substrates other than glucose in the complex medium. This would be in accord with the high overall recovery of carbon in the products.

Fig. 1. *Streptococcus pleomorphus* strain EBF61/60B: (a) grown in VL broth for 24 h; (b) grown in SM10 broth for 24 h (using the Hungate technique); (c) grown on a BGPhf agar plate for 3 days (under hydrogen containing 10% CO₂). Bar markers represent 5 μ m.

The fermentation products formed by the other nine strains, including the group VIIa strain (NCTC9808) were examined by semi-quantitative methods. The earlier tests involved the use of paper chromatography (Barnes & Impey, 1968) whilst subsequent tests were carried out using gas-liquid chromatography (Barnes & Impey, 1974). In all strains lactic acid was the major product; small amounts of butyric and formic acids were found and in some strains acetic acid was also detected. Quantitative examination of the lactic acid produced by all these strains showed that it was always the L(+) form and accounted for 50 to 80% of the glucose carbon utilized according to the strain.

Table 1. *Properties of 10 strains included in the new species S. pleomorphus*

	Hare group VIIa (NCTC9808)	Group 3 peptostrepto- cocci	Type strain EBF61/60B (NCTC11087)
No. of strains	1	9	1
Anaerobic growth	+	+	+
Growth in air or air + 10 % CO ₂	—	—	—
Growth at			
45 °C	NT	+	+
37 °C	+	+	+
20 °C	NT	—	—
Catalase	—	—	—
Weak β -haemolysis (VL blood agar)	—	+	+
Glucose fermentation (L-lactic acid as the sole major product)	+	(4/9)*	+
Terminal pH in glucose broth	4.6	4.4–5.0	4.6
Copious gas	—	—	—
Growth in basal medium without carbohydrate	—	—	—
Acid from			
Arabinose, cellobiose, dextrin†, galactose, lactose, maltose, mannitol, inositol, salicin, starch, sucrose or xylose	—	—	—
Fructose	+	+	+
Mannose	v	+	+
Indole production	—	—	—
Nitrate reduction	—	—†	—
Weak H ₂ S production	+	+	+
Gelatin liquefaction	—	(8/9)	—
Cysteine milk	No change	No change	No change
Growth in presence of			
Brilliant green (1/100 000)	NT	+	+
Kanamycin (100 μ g ml ⁻¹)	+	†	+
Neomycin (100 μ g ml ⁻¹)	+	†	+
Polymyxin B (10 μ g ml ⁻¹)	NT	+	+

NT, Not tested; +, positive; —, negative; v, variable for the same strain.

* No. of strains giving the reaction shown.

† Only four strains of the group 3 peptostreptococci were tested.

Table 2. *Products of the fermentation of glucose by S. pleomorphus strain EBF61/60B*

Product	Amount formed [mmol (mol glucose fermented) ⁻¹]*
Carbon dioxide	124
Formic acid	114
Acetic acid	8
n-Butyric acid	64
Lactic acid	2000
Succinic acid	10
Molar growth yield [g dry wt (mol glucose) ⁻¹]	25
Carbon recovery (%)	109

* All the glucose initially present was utilized and no products other than those indicated were detected.

Table 3. *Differential properties of S. pleomorphus* sp.nov., *S. hansenii*, *S. intermedius*, *S. constellatus* and *S. morbillorum*

	<i>S. pleomorphus</i>	<i>S. hansenii</i> *	<i>S. intermedius</i> †	<i>S. constellatus</i> *	<i>S. morbillorum</i> *
Aerotolerance	—	—	+	+	+
Acid from					
Cellobiose	—	—	+	w—	—
Dextrin	—	—	+	w—	—
Fructose	+	—	+	+	—
Galactose	—	+	+	+	—
Glucose	+	+	+	+	w
Lactose	—	+	+	—	—
Maltose	—	+	+	+	w
Mannose	+ or —	—	+	+	w
Salicin	—	—	+	+	—
Starch	—	—	—	+	—
Sucrose	—	—	+	+	w
Cysteine milk	No change	Clot	Acid, clot	No change	No change
H ₂ S production	w	+	—	—	—

+, Positive (for acid formation, pH below 5.5); w, weak positive (for acid formation, pH 5.5 to 6.0); —, negative; w—, variable for the same strain.

* Type strains of Holdeman & Moore (1974).

† Type strain (Holdeman & Moore, 1974) and strains EBF77/25 and NCTC9817.

DNA base ratio

Four determinations on DNA from strain EBF61/60B gave T_m values of 85.9, 85.6 and 85.2 °C (average 85.5 °C) corresponding to a GC content of 39.4 mol %, compared with a T_m value of 90.9 °C for *E. coli* B (GC content 52.8 mol %).

DISCUSSION

The results indicate that the organisms previously designated group 3 peptostreptococci (Barnes & Impey, 1970) together with the group VIIa anaerobic coccus (NCTC9808) of Hare (1967) belong to the genus *Streptococcus* rather than to the genera *Peptostreptococcus*, *Ruminococcus* or the recently described *Coprococcus* (Holdeman & Moore, 1974) because of their homolactic fermentation with the production of L-lactic acid. The GC content of the DNA (39 mol %) is within the range (32 to 42%) reported for other members of the genus (Deibel & Seeley, 1974). The organisms are strict anaerobes, all the strains isolated so far being unable to grow on an optimal growth medium in air or air containing 10% CO₂. In this and other respects they are unlike *Streptococcus intermedius*, *Streptococcus constellatus* or *Streptococcus morbillorum* now classified by Holdeman & Moore (1974) in the genus *Streptococcus*, and also differ from the new anaerobic species *Streptococcus hansenii* described by Holdeman & Moore (1974). They are considered sufficiently different from these four species to warrant the creation of a new species named *Streptococcus pleomorphus*; some of their differential properties are shown in Table 3. Apart from the unusual morphology, other differences include the failure of *S. pleomorphus* to ferment sucrose, maltose, galactose and lactose.

The nine strains isolated from poultry and previously designated group 3 peptostreptococci, together with the group VIIa anaerobic coccus (NCTC9808) isolated by Hare (1967) from human faeces, belong to the proposed new species *S. pleomorphus* described below.

Streptococcus pleomorphus sp. nov.(pleo.mor'phus. Gr. adj. *pleomorphus* many forms)

Pleomorphic cocci, occurring singly, in pairs and short chains; size and shape may vary considerably according to the medium and conditions of growth (Fig. 1). Gram-positive in young cultures, often becoming Gram-negative within 24 h. On optimal growth media such as RCM, BGPhlf or VL agar, after incubation for 3 days, colonies are about 2 to 3 mm in diameter, circular, convex and with an irregular edge. On VL blood agar some strains may be weakly β -haemolytic. Growth in broth is flocculent.

Anaerobic, no growth occurring in air or air containing 10 % CO₂. No growth at 20 °C, good growth at 37 and 45 °C. Catalase negative.

Carbohydrates are required for growth. The terminal pH varies between about 4.4 and 5.0. Glucose is fermented by homolactic fermentation to L-lactic acid. Gas is not produced. Traces of butyric, formic and sometimes acetic and succinic acids are also found.

Acid is produced from glucose, fructose and usually mannose. No acid is produced from arabinose, cellobiose, dextrin, galactose, inositol, lactose, maltose, mannitol, salicin, starch, sucrose or xylose. There is no change in cysteine milk, gelatin is not liquefied, indole is not produced, and nitrates are not reduced. Small amounts of H₂S are detected in media containing ferrous sulphate and sodium thiosulphate.

Growth occurs in the presence of polymyxin B (10 µg ml⁻¹), neomycin (100 µg ml⁻¹), kanamycin (100 µg ml⁻¹) and most strains are resistant to brilliant green (1/100 000).

Source: the intestines of poultry and occasionally human faeces.

Type strain. NCTC11087 (= EBF61/60B). The GC content of the DNA of the type strain is 39 mol % (by *T_m*).

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