SHORT ARTICLES

AN UNUSUAL BACTERIUM ISOLATED FROM A CHRONIC THROAT INFECTION

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AN unusual bacterium, incapable of growth in fluid medium, was isolated from a case of chronic sore throat in a young woman. It could not be classified, and possessed characters indicating relationship with *Streptococcus*, *Corynebacterium* and *Streptobacillus*. Penicillin treatment had been without effect.

MATERIALS AND METHODS

Organisms. The original strain (designated RR in the following account) was isolated on several occasions from the tonsillar surface, by means of cotton-wool swabs cultured on horse blood agar plates. Variant forms, derived from it in culture, are designated C.

Culture methods. For isolation and subsequent investigation, aerobic cultures were made on blood agar and Fildes agar based on Oxoid Blood Agar Base no. 2. Blood agar plates were made with 10 per cent. horse blood agar over base agar. Fildes extract (Cruickshank, 1965) was added to agar or Oxoid Nutrient Broth, where stated, in a final concentration of 1 or 2 per cent. v/v. Cultures were incubated at 37°C.

Because organism RR did not grow in fluid medium, physiological tests were made on solid medium or on suspensions. For the latter purpose the organism was grown either on 1 per cent. Fildes agar, in a layer over base agar in 30 × 40 cm vitreous enamel dishes, and harvested by scraping; or in 200 ml 1 per cent. Fildes broth in a Winchester bottle, rotated at 10 r.p.h. (House and Wildy, 1965). The bacteria formed colonies on the glass, and were harvested by shaking and centrifugation. In both methods, contamination was controlled by visual and microscopic examination, and by subculture. Contaminated suspensions were rejected.

Indole and ammonia production were tested for by adding 2 ml of bacterial suspension (Brown's opacity tube 10) in 1 per cent. Fildes broth, to a further 2 ml of broth in 10-ml bijou bottles, rotated at 33 r.p.h. (Jeynes, 1961). Tests were made at intervals of 24 hr up to 168 hr, using Kovacs' reagent for indole, and Nessler's reagent for ammonia. Controls with *Escherichia coli* and *Aerobacter aerogenes* were included. For nitrate reduction, 0.1 per cent. w/v potassium nitrate was added to the broth; tests were made with Griess-Ilosvay reagent. Gelatin liquefaction was tested for by the method of Frazier (1926), on 1 per cent. Fildes agar with 0.5 per cent. w/v gelatin; catalase, on growth transferred from 1 per cent. Fildes agar to microscope slides; and oxidase, on growth transferred to filter-paper strips soaked in Kovacs' reagent.

Carbohydrates were sterilised by membrane filtration (Millipore Ltd). Fermentation was tested by three methods: (a) on 1 per cent. Fildes agar at a final carbohydrate concentration of 1 per cent. w/v; (b) on 1 per cent. Fildes agar, with 6-mm disks of sterile filter-paper soaked in a 10 per cent. w/v solution of the carbohydrate, and placed on the surface after inoculation of the bacterial suspension; (c) in a suspension of bacteria diluted to Brown's opacity tube 10 with 1 per cent. Fildes broth, containing carbohydrate at a final concentration of 1 per cent. w/v, incubated with shaking in a waterbath. In all cases, bromthymol blue was added as an indicator after 24–168 hours' incubation.

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Conventional physiological tests were also performed with the C variants, which were able to grow in fluid medium containing 1 per cent. Fildes extract.

Auxanography was performed on 1 per cent. Fildes agar plates, on the surface of which very small quantities (single crystals where practicable) of additive were placed after inoculation. The X factor (haematin) and V factor were tested on nutrient agar with Mast disks.

Soluble haemolysin was tested conventionally, and also in extracts prepared by ultrasonic disintegration and centrifugation at 40,000*g* for 10 min. The supernatant was mixed with an equal quantity of 5 per cent. washed sheep or horse RBC in saline, and incubated at 37°C in a waterbath.

Sensitivities. Antibiotic sensitivity was tested with disks (Mast Laboratories Ltd), and the zone of inhibition measured after 24 hours' incubation.

Sensitivity of isolated walls to lysozyme digestion was investigated by incubating a suspension of purified cell walls (Salton, 1964) with lysozyme. The reaction mixture contained 0.2 mg per ml crystalline lysozyme (40,000 units per mg, BDH) in phosphate buffered physiological saline at pH 7.0. The suspension was incubated at 37°C and the optical density was compared with a lysozyme-free control after 30 min.

All tests were performed in multiples of 3, or more if readings were required to be made at intervals.

Chemical analysis. For cell wall analysis, cells were disintegrated ultrasonically, at a power of 200 W at 20 kHz, in a bath of ice-water, and harvested in an ultracentrifuge at 40,000*g* for 20 min. The sediment was processed according to Cummings and Harris (1956), with the addition that amino acid hydrolysates were precipitated by addition of concentrated ammonia solution, centrifuged and re-evaporated. Hydrolysates were finally dissolved in 10 per cent. v/v isopropanol, and descending-front paper chromatographs of amino acids were performed on Whatman no. 1 or 542 papers, with as solvent isopropanol: acetic acid: water, 70:10:20. For two-dimension chromatography, the second solvent was lutidine: water, 65:35, in an atmosphere equilibrated against 10 per cent. aqueous dimethylamine; or phenol: water, 80:20 with 0.008 per cent. w/v 8-hydroxyquinoline. The papers were then dried, steamed for 10 min., redried and developed by means of a ninhydrin spray {0.2 per cent. w/v tri-ketohydrindene hydrate (BDH), 48 per cent. v/v *n*-butanol, 50 per cent. v/v acetone, 1 per cent. v/v water, 1 per cent. v/v collidine}, and heated at 105°C for 5 min. in a hot-air oven. Standard markers (Shandon Scientific Co.) were employed.

Descending-front paper chromatographs of sugar hydrolysates were performed according to Cummings and Harris.

Antigenic analysis. Extracts for precipitin tests were prepared and tests performed by Lancefield's and Fuller's methods, according to Cruickshank, against streptococcal typing sera and diphtheria antitoxin (Burroughs Wellcome). Tests were also made by gel-diffusion precipitation (Ouchterlony, 1948) and by the method of Elek (1948) against living organisms on 1 per cent. Fildes agar.

Pathogenicity. Pathogenicity of the organisms to mice was examined by the intra-peritoneal injection of suspensions of live organisms. A suspension equivalent to Brown's tube 2 in opacity was prepared in phosphate buffered saline pH 7.2 and two groups of five C₃H albino male mice received 0.1 and 0.2 ml respectively.

A further group of five mice (C₃H females) received 0.1 ml by the same route and these were killed after 24 hr and the peritoneal cavities washed out with saline. Each suspension was centrifuged to sediment all cells, and the deposit was resuspended in 0.5 ml saline and inoculated on a blood agar plate. Each mouse was examined separately. The plates were incubated for 48 hr at 37°C and examined.

Microscopy. Wherever possible living material was examined by phase-contrast and dark-ground microscopy with the Heine optical system (E. Leitz G.m.b.H.) with $\times 90$ h objective, NA 1.15 oil immersion. Heat-fixed smears were stained by Gram's method. Cell walls were stained either by the tannic acid-violet method (Gutstein, 1926; Robinow, 1945) or by the phosphomolybdic acid-methyl green method (Hale, 1953). Impression

preparations of colonies were made by the agar-block fixation method (Klieneberger and Smiles, 1942) and stained with Giemsa.

Electron-microscope examination was made by suspending organisms from plate cultures in 10 per cent. v/v isopropanol. After a brief period of standing to allow gross clumps to settle, the supernatant was diluted until it was just visibly turbid and drops of this were placed on Formvar (poly-vinyl-formol) coated copper grids (Schaefer and Harker, 1942),

TABLE I
Cultural characters of variants RR and C

Character		Organism	
		RR	C
Production of	Soluble haemolysin (horse or sheep red cells)	+	—
	Catalase	+	+
	Oxidase	—	—
	Indole	—	—
	Ammonia	—	—
	Reduction of nitrate	—	—
	Liquefaction of gelatin	—	—
	Acid-fastness to 1 per cent. H ₂ SO ₄	—	—
Carbohydrates—	Fructose	⊥	⊥
	Sucrose	⊥	⊥
	Glucose	⊥	⊥
	Maltose	⊥	⊥
	Galactose	⊥	—
	Mannose	⊥	⊥
	Lactose	⊥	⊥
	Trehalose	V	⊥
	Melibiose	V	—
	Raffinose	V	—
	Cellobiose	V	⊥
	Dextrin	V	⊥
	Glycerol	V	—
	Erythritol	—	—
	Rhamnose	—	—
	Arabinose	—	—
	Inulin	—	...
	Dulcitol	—	—
	Salicin	—	...
	Inositol	—	—
	Xylose	—	—
	Mannitol	—	—
	Sorbitol	—	—

⊥, Acid produced; —, no acid produced; V, variable; ... not tested.

shadowed with gold-palladium alloy 60:40 at a ratio angle of 3:1 and examined in an EM 6 electron microscope (Associated Electrical Industries Ltd) at an accelerating potential of 75 kV.

Organisms were also grown on collodion membranes on the surface of 2 per cent. v/v Fildes agar and incubated at 37°C. Areas of membrane were floated off and mounted on copper grids.

RESULTS

Primary colonies of organism RR on blood agar resembled a β -haemolytic streptococcus; and were composed of small, Gram-positive cocci. On subculture it remained haemolytic, but in morphology, colony appearance and production of L-form colonies, began to

resemble *Streptobacillus moniliformis* (Breed, Murray and Smith, 1957; Wilson and Miles, 1964).

Growth could be obtained on blood agar or Fildes agar (1 or 2 per cent.), but not on nutrient agar, nutrient gelatin or serum agar, and only within narrow limits around 37°C, at which temperature it survived for only 24 hr, and for a shorter time as the temperature varied from the optimum. After repeated subculture, survivals of up to 10 days at 37°C, and for a rather shorter time at 4°C were recorded. Aerobic and anaerobic growth and survival were similar. In fluid media, colonies formed only on the glass surface of rotated bottles partially filled with Fildes broth, but not on the base or walls of stationary vessels. Colonies were formed equally well in an atmosphere of air, nitrogen (with or without 5 per cent. carbon dioxide), or hydrogen.

TABLE II
Antibiotic and drug sensitivity

Antibiotic or drug	Concentration	Inhibition zone diameter for organism	
		RR	C
Ampicillin	25 µg per disk	29	20
Bacitracin	8 units per disk	0	19
Chloramphenicol	25 µg per disk	27	28
Colistin methane sulphonate	200 µg per disk	20	18
Fusidic acid	10 µg per disk	0	25
Kanamycin	30 µg per disk	25	25
Lincomycin	2 µg per disk	0	23
Methicillin	10 µg per disk	30	13
Neomycin	10 µg per disk	13.5	23
Noxythiolin	5 mg per disk	29	35
Nystatin	50 units per disk	0	7
Paromomycin	10 µg per disk	16	23
Penicillin G	1 unit per disk	0	23
Polymyxin B	100 units per disk	13	14
Streptomycin	10 µg per disk	15	25
Tetracycline	25 µg per disk	20	27
Optochin	5 µg per disk	0	0
Lysozyme	0.2 mg per ml	(Sensitive)	(Sensitive)

Satellitism to colonies of staphylococci was noted, but auxanographic tests were negative. No enhancement of growth was produced by X, V or X+V factor disks, or by sodium deoxyribonucleate, coenzyme A, triphosphopyridine nucleotide, adenosine triphosphate, adenosine diphosphate, flavine adenine dinucleotide or α - ϵ -diaminopimelic acid.

The C variants appeared spontaneously, on repeated occasions, in cultures on solid media. Four strains were isolated and examined. The colonies were similar to those of RR but non-haemolytic; they consisted of very small Gram-positive cocci in chains, with irregular small rods, often having clubbed ends. They were capable of growth on nutrient agar, although this was enhanced by blood or Fildes extract. In fluid media they produced uniform turbidity, and did not form colonies on glass in rotated cultures. The optimum temperature range and survival were similar to those of the parent organism.

Morphologically, both in cell-wall stained preparations and in the electron microscope, RR forms showed a very distinctive feature. The rods or filaments had small, coccal terminal bodies, as well as the pleomorphic forms, often with weakened cell walls, characteristic of streptobacilli. The C variants appeared as simple cocci.

Cultural characteristics of the two variants are shown in table I; sensitivity to drugs and antibiotics, in table II; amino acids detectable by chromatography, in table III. The

sugars detectable in RR variants were arabinose and galactose; rhamnose was absent. Clear results for sugar analyses of C variant walls were not obtainable by the methods employed; the reason for this is not known.

Serological tests were all negative. No precipitation was observed against any commercially available anti-streptococcus sera, whereas controls against known streptococci were positive. The Elek plate tests against diphtheria antitoxin were negative; positive controls gave strong reactions.

All mice survived the pathogenicity tests, and cultural examination showed the peritoneal cavity to be sterile 24 hr after infection.

After a course of treatment with chloramphenicol the sore throat ceased, and organism RR could not thereafter be cultured from the patient, although the remainder of the flora of the throat was unaltered.

TABLE III
Cell-wall analysis

Amino acid	Presence (+) or absence (—) in organism	
	RR	C
Diaminopimelic acid	—	+
Lysine	+	Tr*
Glycine	+	+
α-Alanine	+	+
Valine	+	+
Leucine	+	+
Aspartic acid	+	+
Glutamic acid	+	+
Threonine	—	Tr
Ornithine	—	—

* Tr = Trace.

DISCUSSION

Organism RR must be regarded as a variant phase of a small Gram-positive organism. Few reports of comparable isolates from pathological conditions have been made. The "transitional forms" of corynebacteria (Wittler *et al.*, 1960) are similar but not identical.

One of the most unusual features of organism RR is that it cannot grow in fluid media, but forms colonies on glass surfaces, after the fashion of tissue cells.

By the criteria of Cowan and Steel (1965) and Breed *et al.* (1957) this organism can be regarded as related to *Corynebacterium*, *Streptococcus* and *Streptobacillus*. Cell-wall analysis suggests a relationship with *Corynebacterium* and *Streptococcus* (Cummings and Harris, 1956), the sugars being characteristic of the former, and the amino acids of the latter, except for diaminopimelic acid (in C variants), which is suggestive of *Corynebacterium*, and has also been found in an oral diphtheroid (Davis and Freer, 1960).

The sore throat ceased with the elimination of the organism, which may thus be considered at least to have contributed to it.

It may be assumed that RR was a variant form, induced by penicillin therapy, and that the C variants represented a return towards the normal bacterial phase.

SUMMARY

The characteristics of a Gram-positive bacterium isolated from a chronic sore throat are described. It possessed a streptobacillary morphology and was capable of growing

only upon a solid surface. Its physiology and chemical composition, and those of Gram-positive variants derived from it, suggest a relationship with both *Streptococcus* and *Corynebacterium*, especially the latter.

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THE ANTIVIRAL ACTIVITY OF ISOQUINOLINE DRUGS FOR RHINOVIRUSES *IN VITRO* AND *IN VIVO*

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CERTAIN isoquinoline compounds have been found to have antiviral activity *in vitro* (Brammer, McDonald and Tute, 1968). Larin *et al.* (1968) showed that two such derivatives of dihydroxyisoquinoline, namely 1-(*p*-chlorophenoxymethyl)-3,4-dihydroisoquinoline hydrochloride (UK2054) and 1-(*p*-methoxyphenoxymethyl)-3,4-dihydroisoquinoline hydrochloride (UK2371) showed activity *in vitro* against a range of viruses, including myxoviruses and

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