Cultural Characters of *Streptococcus pluton* and its Differentiation from Associated Enterococci

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SUMMARY

Strains of *Streptococcus pluton* (White) from widely separate parts of the world were very similar culturally and serologically and differed greatly in these respects from associated enterococci. *S. pluton* was satisfactorily isolated only on a yeast + glucose + potassium phosphate + starch medium incubated anaerobically + CO₂. Oxidation potentials in agar of this medium were very stable for which the high concentration of phosphate, together with yeast and glucose, was necessary. In deep agar incubated aerobically, *S. pluton* started to grow only within a narrow range of Eh values. It did not grow on ordinary bacteriological media but was adapted to grow on the special agar in air + CO₂. Adapted strains grew on the special agar in a limited volume of air and produced CO₂.

INTRODUCTION

Since the first account of the isolation and cultural characteristics of *Streptococcus pluton* (White) from honey bee larvae with European foul brood disease (Bailey, 1957), further work on several isolates from different parts of the world has been done. Information was sought that would facilitate the differentiation of *S. pluton* from organisms resembling *Streptococcus faecalis*, which often occur in diseased larvae and are still confused by bacteriologists with *S. pluton*.

METHODS

Mid-guts of living diseased larvae were removed aseptically, smeared on sterile microscope slides, covered, allowed to dry and stored at room temperature. *Streptococcus pluton* stays viable in such smears for at least 15 months and associated bacteria do not (Bailey, 1959). Material of this kind was collected from Southern England (E); Wisconsin (W), New York (N) and Louisiana (L) U.S.A.; Argentina (A) and Switzerland (S); strains from these sources are referred to below by the single letter. One strain from each source and an additional unusual strain, P, from England were tested.

The usual medium, referred to as 'basal' below, was as follows: 1 g. yeast extract (Difco); 10 ml. M-KH₂PO₄ adjusted to pH 6.6 with KOH; 2 g. agar; made up to 100 ml. with distilled water. Ten ml. lots were distributed into screw-capped bottles and autoclaved at 116° for 20 min. immediately before use.

Unless otherwise stated, cultures were incubated anaerobically at 34° in McIntosh and Fildes jars containing hydrogen + about 10% (v/v) CO₂.

Organisms identified as *Streptococcus faecalis* were isolated from diseased larvae

from Argentina, Wisconsin (U.S.A.) and Switzerland: strains A', W', and S₁, S₂, S₃, S₄, respectively.

Failure to grow on modified or different media is reported only when similar inoculation gave growth on a suitable medium.

Antisera were prepared by injecting rabbits with washed formalized suspensions of strains N and W'. Four intravenous injections were given during 14 days, and the rabbits bled 12 days after the last injection. Serological assays were made by agglutination tests in tubes containing suspensions of bacteria equiv. 0.25 mg. dry wt./ml. Qualitative tests were made by a micro gel-diffusion method (Mansi, 1958) with bacterial suspensions and with extracts made from them by the method described by Lancefield (1933).

RESULTS

Cultural characters

For growth of *Streptococcus pluton*, basal medium stored for a few weeks was inferior to basal medium freshly prepared, and the bacteria that grew on it were very pleomorphic, sometimes being mostly rod-like. Basal medium (100 ml.) + 0.5 g. Neopeptone (Difco) deteriorated quicker than did basal medium alone. Growth in pour plates without starch sometimes failed even when streaks on similar plates with the same inoculum produced colonies. Many of the organisms which did grow in pour plates were rod-like. Good growth occurred in pour plates of basal medium + starch. The easiest way to isolate *S. pluton* from natural material containing enterococci was to inoculate such pour plates with decimal dilutions of bacterial suspensions. Enterococci were fewer than *S. pluton* even in diseased larvae which had decomposed in a moist state for some days, and were easily diluted out. Colonies of enterococci were larger than those of *S. pluton* and were surrounded by a clear halo. Small inocula of *S. pluton* grew well in pour plates of basal medium + starch; most of the organisms grew from inocula containing about fifty bacteria. Slopes of basal medium were superior to plates or broths for subculturing *S. pluton*, growth often appearing within 2 days.

Many attempts were made to isolate the aerobic rod-forms observed with the first isolates (Bailey, 1957) from all sources of *Streptococcus pluton*, but all failed. Growth often occurred in aerobically incubated broths, particularly in narrow or sealed tubes, but it was in streptococcal form or in chains of mixed rods and cocci that could not be subcultured aerobically on the surface of nutrient agar. Nevertheless strains tested adapted to oxygen when they were subcultured on slopes of basal medium in an atmosphere of (% v/v): 5 air + 85 H₂ + 10 CO₂ in sealed 3 l. jars. They could then be subcultured on 10 ml. slopes in tubes sealed with about 20 ml. of air (in which they produced CO₂) or on slopes in the large jars with up to 90 % (v/v) air + 10 % (v/v) CO₂. No growth occurred in the large jars without CO₂.

The growth of *Streptococcus pluton* in deep agar media (Table 2) was the same with inocula from dried smears or from pure cultures. The concentration of the inoculum made little difference to the depths at which growth appeared. The upper limit of the lower bands appeared at the level where methylene blue (2 mg./l.) incorporated in similar sterile incubated media was reduced. This boundary of reduction was very stable: in deep agar tubes of basal medium it was about 10 mm. from the surface and moved down to 15 mm. after aerobic incubation for 3 weeks. In basal medium,

Table 1. *Growth of Streptococcus pluton and associated enterococci in various media at 34°*

Media constituents and environmental conditions*	Growth**												
	<i>S. pluton</i>							Enterococci					
	S	E	W	N	L	A	P	Strains A' W' S ₁ S ₂ S ₃ S ₄	Remarks	Growth within 24 hr. Final pH 4.0			
60° for 30 min. before incubating	+	+	+	+	+	+	+		Growth after 2 or more days. No acid				
0.5 % Na taurocholate	+	+	+	+	+	+	+		Primary isolates sometimes failed				
Ordinary media†	+	+	+	+	+	+	+		Type P strains were rare in natural material				
Litmus milk	+	+	+	+	+	+	+		Only 1 of 2 replicates of L grew				
10 % CO ₂	+	+	+	+	+	+	+		Primary isolates feeble. Final pH 5.2				
Anaerobic	+	+	+	+	+	+	+		Training necessary. Catalase negative				
Aerobic	+	+	+	+	+	+	+						
0.2 M-NaCl	+	+	+	+	+	+	+						
0.1 M-NaCl	+	+	+	+	+	+	+						
0.1 M-KCl	+	+	+	+	+	+	+						
0.1 M-KHCO ₃ + 75 % v/v CO ₂ (pH 6.6)	+	+	+	+	+	+	+						
0.1 M-Na phosphate, pH 6.6	+	+	+	+	+	+	+						
0.1 M-K phosphate, pH 9.0	+	+	+	+	+	+	+						
0.01 M-K phosphate, pH 6.6	+	+	+	+	+	+	+						
0.1 M-K phosphate, pH 6.6	+	+	+	+	+	+	+						
1 % starch	+	+	+	+	+	+	+						
1 % glucose	+	+	+	+	+	+	+						
1 % yeast extract (Difco)	+	+	+	+	+	+	+						

*, medium constituent or environmental condition present.
 **, +, strong; +, moderate; ±, slight; —, nil.
 † Nutrient broth no. 2 (Oxoid) or brain heart infusion (Difco).

with glucose added after autoclaving, methylene blue appeared less abruptly reduced at a depth of about 20 mm., and the boundary moved down about 10 mm. after incubation for 3 weeks. The yeast extract, glucose and phosphate each contributed substantially to the stability of the oxidation/reduction potential; starch in the medium made no difference. In basal medium + 5 g. Neopeptone (Difco)/l. the boundary of methylene blue reduction was deeper and less well defined than in ordinary basic medium. (The phosphate and glucose autoclaved together without yeast yielded a well-poised agar: methylene blue was reduced at 15–20 mm. below the surface with no change after a week.) Media containing Nile blue remained blue to the bottom of the

Table 2. *Growth of Streptococcus pluton and associated enterococci in deep agar**

	Medium	<i>Streptococcus pluton</i> (strains, E, L, N)†	Enterococci (all strains)
(1)	g./l.: yeast extract (Difco), 10; glucose, 10; 0.1 M potassium phosphate (pH 6.6); autoclaved in screw-capped bottles at 116° for 20 min.	Colonies after several days from 3 to 8 mm. below surface, trailing to variable depths. Thin band (< 1 mm) of optimal growth at upper level, sometimes with a second similar band about a mm. above. Growth sometimes feeble or absent	Strong, even growth from surface to bottom within 24 hr.
(2)	(1) with glucose autoclaved separately	Colonies after several days from 10 to 25 mm. below surface, trailing to variable depths, and usually optimal at the upper level; followed by a second band a few mm. above, ending a few mm. below surface and usually optimal at its lower level. A third band occasionally appeared over the second. Growth sometimes feeble or absent	As above
(3)	As (1) or (2) but autoclaved in open tubes	As in (1) or (2) but growth restricted to the optimal regions	—
(4)	(1), (2) or (3) + 10% v/v CO ₂ in air	Little change in (1) or (2); growth restrictions observed in (3) removed	—
(5)	(1) or (2) + starch	As in (1) or (2) but optima and banding less distinct although growth more reliable	—
(6)	(1) + Neopeptone (Difco)	As in (1) but with wider single band of optimal growth	—

* Molten agar media inoculated, poured into tubes and incubated aerobically at 34°.

† See text.

tube. *S. pluton* grew well in deep basal agar with methylene blue and double-banded growth occurred as shown in Table 2. When deep basal agar tubes which had been inoculated and incubated aerobically for 2 days were extruded from their tubes before growth was visible and were then re-incubated aerobically, colonies appeared in the position that the lower band of growth normally occupied in deep agar tubes. Such deep basal agars with methylene blue and inoculated with *S. pluton* became blue throughout when extruded.

Wille (1960) reported difficulty in culturing *Streptococcus pluton*, much of which was undoubtedly caused by absence of CO₂ (he used alkaline pyrogallol to achieve anaerobiosis) and starch. He reported that added trace elements (1% v/v Hoag-

land solution Hoagland & Snyder, 1931) improved matters. Hoagland solution was tried with all the various strains of *S. pluton* except W but did not improve growth in the basal medium, with or without starch, on plates or slopes, or in liquid media and did not cause growth in such media incubated without CO₂.

Serology

The serological tests confirmed the results of the cultural tests described above. The type of immunizing antigen used and the timing of the injections resulted in antisera which contained predominantly 'type' antibodies. Agglutination tests showed that all *Streptococcus pluton* isolates were serologically related to one another but not to the associated enterococci (Table 3). Not all the enterococci were similar; the strain W' antiserum reacted with strain W' and strain A', but not with strains S₁₋₄.

As the antisera contained few group antibodies, the lines of precipitate formed in gel diffusion tests were usually very faint, even when concentrated preparations of antigen and antiserum were used. The results, however, confirmed those of the

Table 3. *Agglutination tests of bacterial isolates*

Antigen: bacterial suspension 0.25 mg./ml.

Antigen	Antiserum titre*	
	Strain N antiserum	Strain W' antiserum
<i>S. pluton</i> strains		
E	1024	< 2
W	512	< 2
N	1024	< 2
L	256	< 2
A	512	< 2
P	1024	4
S	1024	8
<i>S. faecalis</i> strains		
A'	< 2	256
W'	4	1024
S ₁ , S ₂ , S ₃ , S ₄	< 2	< 2

* Reciprocal of dilution end point after incubation for 4 hr. at 37°.

agglutination tests. Both antisera sometimes gave a faint line of precipitate, in the gel close to the antiserum well, against all isolates; this line was clearest when suspensions of bacteria were used, and was indistinct or absent when extracts were used. In addition the strain N antiserum gave a second faint line of precipitate against concentrated extracts of *Streptococcus pluton* isolates, and the strain W' antiserum gave a dense line of precipitate against strain W' extracts, a weaker line against strain A' extracts, and sometimes a faint line against strain S extracts. Strain W' antiserum reacted with strains A' and W' in gel diffusion and in agglutination tests, showing that these isolates were closely related serologically. However strain W' antiserum only reacted with strains S₁₋₄ in gel diffusion tests with bacterial extracts, which shows that they were less closely related; perhaps at the group level.

DISCUSSION

The *Streptococcus pluton* strains used were not all tested together in every culture trial, but the only considerable cultural differences seem to be between the rare strain P and the others. In its CO₂ requirement and its growth in deep agar *S. pluton* may seem to resemble *Brucella abortus* (Wilson, 1931). Like *B. abortus* it probably grew in deep agar without an external supply of CO₂ because it produced its own, but, unlike *B. abortus*, the bands of growth of *S. pluton* were little influenced when CO₂ was supplied. *S. pluton*, therefore, seems truly micro-aerophilic. Further, when *B. abortus* gave double-banded growth the lower band appeared later and arose, Wilson concluded, because the medium near the growing organism became alkaline and absorbed CO₂. Double-banded growth of *S. pluton*, however, seemed likely to be caused by over-reduction of the medium above the lower band: the upper band probably appeared where the oxygen diffusing from above counter-balanced reduction from below to maintain a critical Eh value long enough for growth to start. This Eh value is probably in the less negative regions of the range -50 to -100 mV., according to the observations with indicators. That growth sometimes failed to reach the bottom of deep agars, was less in deep agar autoclaved in open tubes than in similar agar autoclaved in closed tubes containing air, and was feebler in deep agar or anaerobic broths autoclaved with glucose, than in the same media with glucose added after autoclaving, was further evidence that growth was inhibited in over-reduced media.

The growth that appeared in extruded deep agars, equivalent to the lower band in tubed agar, suggests that the Eh value is most critical for initiation of growth; once begun growth continued in a more oxidized medium. Growth on plates started successfully in an anaerobic atmosphere because the medium may have been rather stably oxidized while setting after pouring and then was gradually reduced through the range of optimal Eh under anaerobiosis. Growth was quicker on slopes than on plates, perhaps because slopes were less exposed to the atmosphere in preparation, and oxidation may have been nearer the optimum. Frequent failure to grow in pour plates of basal medium may have occurred because it was too irreversibly oxidized below the surface, as suggested by the development of rod-like forms when growth did occur. Basal medium + Neopeptone probably deteriorated quickly because it was less well poised than ordinary basic medium and became too oxidized. The band of growth in deep agars containing Neopeptone was wider than in ordinary basal medium, probably because the zone of optimal Eh for growth initiation was wider. The high potassium phosphate concentration is probably required by the ordinary strains of *S. pluton* to give a high K/Na ratio, but the poisoning effect of phosphate may also be important as media with equivalent concentrations of bicarbonate or chloride seemed inadequate.

Failure to produce fully aerobic forms of *Streptococcus pluton* may suggest that those apparently obtained in the original isolates (Bailey, 1957) were contaminants. This cannot be ruled out but is considered unlikely because the original (basal) medium was developed by using these rod-producing cultures, and rod-like forms often arose from all strains of *S. pluton* exposed to oxygen or when plated on aged (presumably oxidized) media. Successful adaptation to fully aerobic growth may require a combination of conditions of inoculum and medium, obtained by chance

in the many changes of medium in the early trials. Some strains more tolerant of oxygen may also have been selected in the early work. The formation of rod-like forms in aerobic conditions, or in oxidized media, may be analogous to the formation of diphtheroid forms in extreme aerobiosis by an α -haemolytic streptococcus (Dubos, 1945).

It is now clear that starch is necessary to ensure isolation of *Streptococcus pluton* from natural material. The need is less with subcultures, however, which undoubtedly led to the original conclusion that it was unnecessary (Bailey, 1957). Presumably starch neutralizes growth inhibitors in the medium, as it does for other fastidious organisms (Foster *et al.* 1950; Ley & Mueller, 1946).

The fastidiousness of *Streptococcus pluton* seems to distinguish it from associated enterococci. In particular the facultative anaerobic growth of enterococci on a variety of ordinary media without CO₂ and their tolerance of sodium separates them sharply from *S. pluton*. Serologically, all the strains of *S. pluton* examined clearly differ from the enterococci. The serological tests indicate that, unlike the enterococci, all strains of *S. pluton* are closely related in spite of their widely separate geographical origins, which probably reflects their specific association with honey bees.

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