THE REACTIONS OF STREPTOCOCCI AND LACTOBACILLI IN BLOOD AGAR

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(With Plate VI and 1 Figure in the Text)

Introduction

The phenomena produced by colonies of streptococci on blood-agar plates have long been used by medical bacteriologists for classification purposes. An examination of the literature showed that only a few contradictory data were available for the corresponding phenomena induced by the saprophytic lactic acid bacteria. A systematic investigation into the types of "haemolysis" and discoloration phenomena produced by typed cultures of lactic acid bacteria was therefore carried out to ascertain whether the blood-agar plate was of any use in the routine classification of these bacteria. The monograph of Brown (1919), which has been generally accepted as the standard work on the subject, is regarded as a standard of reference in the present paper.

The phenomena produced on blood agar fall into two main groups, those of haemolysis and those of discoloration. The latter may vary from yellow to green in colour, and a considerable volume of evidence points to hydrogen peroxide as the causative agent (M'Leod & Gordon, 1922).

The various types of "haemolysis" classified by Brown are caused by combinations of these two mechanisms as shown in Table I.

Table I

Phenomenon	Brown's nomenclature	Soluble haemolysin production	Hydrogen peroxide production
No action	γ	0	0
Greening	(γ green)	0	+
Zone of weak haemolysis at some distance from colony	α prime (α')	+?	0?
Complete haemolysis	β	+	?
Green zone with outer true haemolysis	α	+	+

It may be observed that although "haemolysis" as a phenomenon indicates only a liberation of haemoglobin from the red blood cells, Brown's definition of β haemolysis requires "a sharply defined clear, transparent, colourless, completely haemolysed zone".

The remaining types α and α prime (α') may be considered as true (or β) haemolysis partially obscured by other reactions, such as "greening". For a critical discussion of these factors the reader is referred to Brown's original monograph or to Thomson & Thomson (1927).

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Personal investigations

While in general the saprophytic lactic acid bacteria produce changes in blood agar similar to those described by workers on parasitic types, it was early observed that many strains produced a characteristic appearance, not clearly differentiated by Brown and other workers who investigated pathogenic types. Colonies of these strains produced a round zone of "bleaching" or decolorization of the blood pigment. On examination with the naked eye these colonies might pass for β haemolytic colonies, but on examination with a 15× lens, the corpuscle stromata were seen to be present in (apparently) undiminished numbers (Pl. VI). Examination with a $\frac{2}{3}$ in. objective showed that at the level of the colony the corpuscle stromata were as thickly distributed near the colony as away from it. (Sometimes, however, a perceptible diminution in the concentration of corpuscle stromata would occur either near the periphery of the colony or at a zone some distance from the periphery. It is evident that in the former case, such a colony might justly be described as "very weak β" and in the latter case as "weak α prime".)

In this paper such colonies are described as causing "bleaching".

Technique

Cultures

The cultures used had been isolated from the usual sources or obtained from various laboratories and collections throughout the world. They were repeatedly examined for purity and conformity to type. Strains obviously atypical are not dealt with in this paper.

Medium

10 ml. nutrient agar (1% Hopkins & Williams' peptone, 1% Lab Lemco, 0·5% sodium chloride and 2% agar), 0·5 ml. autolysed yeast (Davis, 1936) and 0·5 ml. defibrinated cow blood.

Autolysed yeast was added to the nutrient agar which was then melted at 100° C., well mixed, cooled to 48–50° C., the blood added and mixed. For poured plates, a loopful of a suitable dilution of a fresh litmus milk or special litmus-milk culture was mixed with the medium, i.e. deep culture.

The blood was taken from healthy cows and had usually been kept in the refrigerator a few days before use. The medium was poured into Petri dishes having an internal diameter of 89–91 mm., so that the average depth of the medium was 1·75 mm. Plates were incubated at the optimum temperature for the strain, except that the "thermophilic" strains were incubated at 37° C. The usual period of incubation was 48 hr., but when the vigour of growth of a strain was such that a shorter or longer period was desirable to obtain colonies from 0·5 to 1 mm. in diameter an incubation period of 1 or 3–4 days was used. Some types of lactic acid bacteria grow very slowly, even on the most

Table II. *Action of streptococci and lactobacilli on blood agar*

(Brown's technique (1919) but with cow blood and autolysed yeast added.)

	Growth	Greening	Bleaching	True haemolysis	Type of haemolysis (Brown, 1919)
		STREPTOCOCCI			
Group I (saprophytes; Davis, 1937, 1939):					
Str. lactis I	+	0	+	0	γ
OJ	+	0	sl.	0	γ
Str. cremoris OJ	0	·	·	·	·
18 OJ	0	·	·	·	·
BDI	0	·	·	·	·
Str. bovis Winsome 2	+	0	+	0	γ
Str. thermophilus OJ	+	+	+	0	γ
670	+	0	0	0	γ
Group II (pathogens and potential pathogens):					
(*a*) *Str.* "*viridans*" (many strains)	+	+	0	?	α
(*b*) *Str. pyogenes* OJ	+	0	0	+	β
2400	+	0	0	+	β
325	+	0	0	+	β
M'Leod	+	0	0	+	β
Str. agalactiae 3481	+	0	0	+	β
Z 62	+	0	0	+	β
Z 169	+	0	+	+?	α′
OJ	+	0	0	0	γ
3444	+	0	0	0	γ
Z 64	+	+	0	0	γ
Mastitis streptococci:					
Minett Group I (non-haemolytic)	+	∓	0	0	γ
I (haemolytic)	+	0	0	+ (hazy)	β weak
II	+	±	0	0	γ
III	+	∓	0	0	γ
Str. pyogenes (animal C)	+	0	0	+	β
(*c*) *Str. faecalis* OJ	+	+	+	0	γ
Z 111	+	+	+	0	γ
2703	+	+	+	0	γ
370	+	sl.	0	0	γ
775	+	sl.	0	0	γ
2707	+	0	0	0	γ
Str. glycerinaceus 2702	+	sl.	0	0	γ
Str. liquefaciens Z 94	+	+	+	0	γ
2705	+	+	0	0	γ
Z 84	+	+	0	0	γ
Z 93	+	+	0	0	γ
Group III (*Leuconostoc*, Van Tieghem; *Betacoccus*, Orla Jensen):					
Leuc. mesenterioides 3274	+	0	0	0	γ
Leuc. mesenterioides X 5	+	0	0	0	γ
Bc. arabinosaceus 2706	+	0	0	0	γ
Str. paracitrovorus D 22	sl.	0	0	0	γ
DN_2	+	0	0	0	γ
Bc. bovis OJ	+	0	sl.	0	γ
Leuc. dextranicum X 22	+	0	0	0	γ
Tetracoccus, Orla Jensen:					
Tc. liquefaciens 3251	+	0	0	0	γ
Tc. casei 2699	+	0	0	0	γ
Tc. mycoides 30 OJ	+	0	0	0	γ

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Table II (*continued*)

	Growth	Greening	Bleaching	True haemolysis	Type of haemolysis (Brown, 1919)
	LACTOBACILLI				
Group I (*Streptobacterium*, Orla Jensen):					
L. plantarum 1–4	+	sl.	+	0	γ
1–8	+	0	sl.	0	γ
2–2	+	0	0	0	γ
3254	+	0	0	0	γ
4125	+	+	+	0	γ
OJ	+	+	0	0	γ
AM	+	+	+	0	γ
11 GS_1	+	+	0	0	γ
3 GS_2	+	0	0	0	γ
12 GS_1	+	+	0	0	γ
L. casei 2–9	+	0	0	0	γ
2–11	+	0	0	0	γ
2–13	+	0	0	0	γ
OJ	+	+	0	0	γ
3253	+	0	+	0	γ
R 12	+	+	+	0	γ
Group II (*Thermobacterium*, Orla Jensen):					
L. helveticus 4113	+	+	+	0	γ
Tbm. Jughurt OJ	+	sl.	sl.	0	γ
Tbm. lactis R 8	+	sl.	sl.	0	γ
R 10	+	sl.	0	0	γ
L. bulgaricus TC	0	.	.	.	.
R 6	0	.	.	.	.
2889	0	.	.	.	.
L. acidophilus 1724	+	0	+	0	γ
1899	+	+	0	0	γ
TC	+	sl.	sl.	0	γ
L. odontolyticus 1406	+	0	+	0	γ
1407	+	+	+	0	γ
Tbm. cereale 4 OJ	0	.	.	.	.
L. Delbrücki B	0	.	.	.	.
4033	+	0	+	0	γ
Group III (*Betabacterium*, Orla Jensen):					
Bbm. caucasicum OJ	0	.	.	.	.
Bbm. longum R 1	0	.	.	.	.
R 2	+	sl.	sl.	0	γ
Bbm. breve R 3	0	.	.	.	.
L. rudensis	+	0	+	0	γ
L. pentoaceticus 947	+	0	0	0	γ
4037	+	+	+	0	γ
L. brassicae fermentatae 4036	+	0	0	0	γ
2108	+	0	0	0	γ
L 28	+	0	0	0	γ
L. gracile Ost.	0	.	.	.	.
Charlton	0	.	.	.	.
L. pastorianus	+	+	+	0	γ
L. acidophil-aerogenes 4034	+	0	+	0	γ
L. bifidus 2797	0	.	.	.	.
Microbacterium, Orla Jensen:					
Mmb. flavum	+	0	0	0	γ
Mbm. lactis	+	0	0	0	γ
Mbm. mesentericum	+	0	0	0	γ

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suitable medium that can be found for them. Thus after 4 days' incubation they can be considered to be at an age physiologically corresponding to more vigorously growing strains after 1 or 2 days.

All plates were examined independently by two or three experienced workers after the incubation period, and again after 24 hr. refrigeration.

The medium described here differs from that usually employed (Brown's medium (Brown, 1919)) in containing autolysed yeast and in the use of cow blood in place of horse blood. Experiments showed that autolysed yeast did not inhibit or engender phenomena obtained with Brown's medium and in many cases permitted growth which, without yeast, was not obtained.

Technique of examination

Plates containing from 200 to 300 colonies were examined first by the naked eye for discoloration. It was found that discoloration was best detected by naked eye examination, holding the plate obliquely in front of a window. If zones of haemolysis or apparent haemolysis were present the colonies were examined under the $\frac{2}{3}$ in. objective.

In poured plates colonies in the depth of the agar were recorded. It was frequently observed that surface colonies gave quite different results.

The results obtained with poured plates are given in Table II.

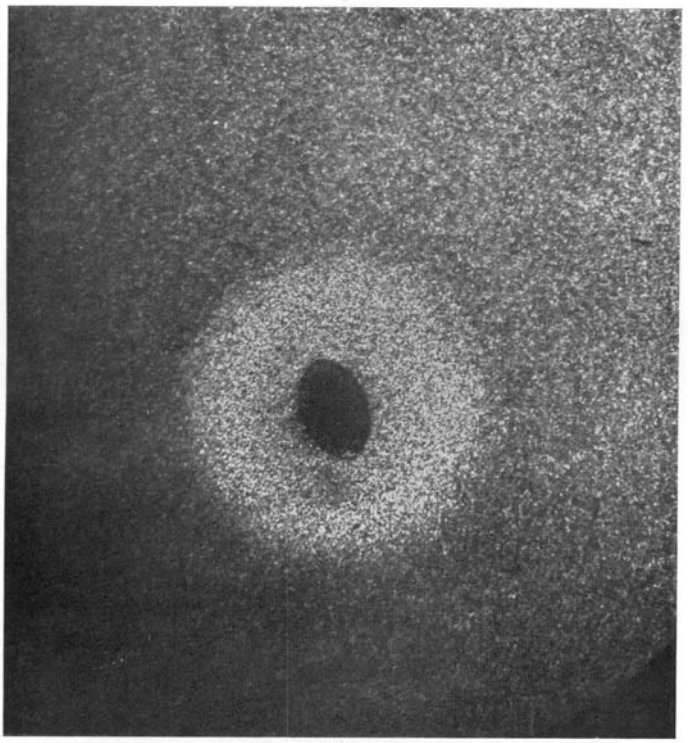
It may be observed that no well-defined correlation exists between species and type of haemolysis on blood agar, in the case of the saprophytic lactic acid bacteria. A certain correlation is observable in the case of the groups as defined in Table III.

Table III

	Greening and bleaching		True haemolysis	
Group	Streptococci	Lactobacilli	Streptococci	Lactobacilli
I	±	∓	0	0
II	0 (*pyogenes*) + (*viridans*)	±	+ (*pyogenes*) ± (*viridans*)	0
III	0	∓	0	0

Thus of all the bacteria listed only some of Group II streptococci produce true (β) haemolysis in blood agar. No lactobacilli are haemolytic although in view of the analogous metabolic behaviour of *Streptococcus* II and *Lactobacillus* II it might be expected that if any *Lactobacillus* is ever found to be haemolytic it would be a member of this group. In addition to a few strains in Group II (rods and cocci) some in Group I produce "bleaching" and so would, on casual inspection, appear to be haemolytic.

Group III cultures are with a few exceptions inert in blood agar. This is in agreement with their respiratory mechanisms (presence of haemin type systems and non-formation of hydrogen peroxide; Davis (1933, 1935)).



Photomicrograph of colony of *Str. lactis* OJ in blood agar showing zone of "bleaching". Magnification: 84 diameters. (Photograph: N. Gruber.)

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Streaked plates

Although Brown (1919) considered poured plates superior to streaked plates, surface colonies have been preferred by some workers. The results obtained with streaked plates on the whole confirmed the results obtained with poured plates.

As a general conclusion it may be said that whereas β haemolysis appears to be a constant phenomenon, independent of respiratory functions and due to a powerful soluble enzyme or lysin produced in the early life of the colony, the other types, viz. α′, "bleaching" and greening, appear to be aerobic in nature and largely controlled by respiratory activity. These may merge one into the other to give ill-defined phenomena. It would appear, therefore, that either the oxidation of haemoglobin prevents true haemolysis (as in the case of types before refrigeration) or that these types are not produced by a true haemolysin but by other substances, possibly end-products of metabolism.

An endeavour was made to differentiate these two kinds of haemolysis (true β haemolysis and other types) by growing strains in lemco broth + 5% cow blood (added aseptically). The results are shown in Table IV.

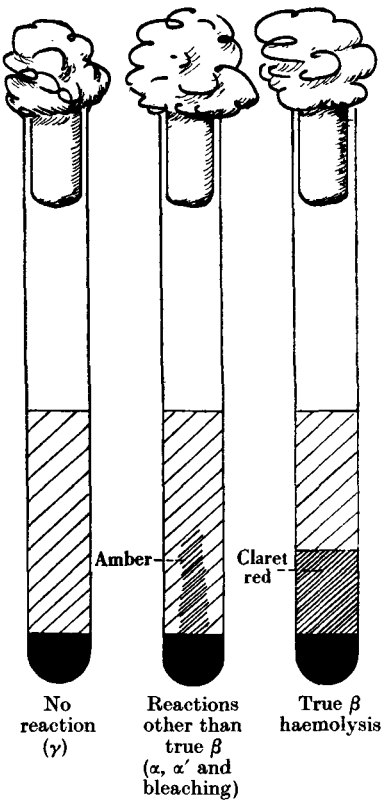
Table IV. *Results after* 24 *hr.* at* 37° (*or* 30°)

Strain	Blood lemco broth (appearance in tube above blood)	Haemolysis type (plate)
Control	—	—
Str. cremoris 18 OJ	—	(No growth)
Str. liquefaciens 2705	Faint amber	Greening
Str. faecalis 370	Faint amber	α′
Str. bovis (?) 2701	Faint amber	α′
L. casei (?) H 831	Faint amber	Greening
L. plantarum 1–8	Faint amber	Slight bleaching
Str. faecalis 775	Amber	Greening
L. helveticus 4113	—	Greening
L. bulgaricus TC	Faint amber	(No growth)
L. pastorianus	Faint amber	Bleaching, greening
Str. agalactiae OJ	Faint amber	Slight greening
3481	Transparent deep claret red	β
Z 62	Transparent deep claret red	β
Z 169	Transparent deep claret red	β
Str. pyogenes Salt	Transparent deep claret red	β
Angel	Transparent deep claret red	β
Str. mitis (?) B 1	Amber	Bleaching, greening
B 3	Amber	Slight bleaching
B 5	Amber	Bleaching, weak β
Str. "*viridans*" 1080	Amber	(? α) greening

* Using as inoculum 2 loopfuls of a yeast dextrose litmus-milk culture truly β-haemolytic strains have given a positive result in as short a time as 3 hr. at 37°.

It will be seen that the reaction in blood lemco broth falls into two distinct groups: (1) those giving at the most an amber coloration in the broth above the blood, and (2) those giving a deep claret red in the broth (Fig. 1). Tests on a large number of strains have shown that "doubtful" reactions are very rare.

This simple tube test is, therefore, a most useful supplementary test to the blood plate when doubt exists as to the type of haemolysis produced by a colony.



Text-fig. 1. The tube test for haemolysis.

Conclusions

Those phenomena produced on blood agar and known as γ, greening (or γ green), "bleaching" and α' (alpha prime) are of no diagnostic value on account of the complexity and variability of the reactions producing these effects. True haemolysis (β) appears to be a constant and diagnostically useful characteristic which is not altered appreciably by differences in technique.

Those types known as α and "weak β" require further study before their value can be appreciated.

The most dangerous aspects lie in the readiness with which "bleaching" merges into α' or "weak β" and in the subjective error in determining whether a "weak β" is β or not. It is suggested that the confusion and disagreement which exists between workers in different laboratories, especially over *Str. agalactiae* may be due not only to the use of blood from different animals and the failure to focus accurately the edge of the colony, but to this merging of

one type into another, and to the ambiguity of the term "weak β". It is recommended that while the blood-agar plate is invaluable for picking out β, "weak β", and other types of colonies, the term β should not be used in describing haemolysis unless the zone is clear ($\frac{2}{3}$ in. objective) and sharply defined. In all cases of doubt (i.e. β or "weak β") a tube test such as the one described should be done. In doubtful cases no strain should be described as haemolytic unless the tube test is positive. Blood plate and tube tests should be recorded side by side in the case of all strains which make any pretence to "β haemolysis".

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REFERENCES

BROWN (1919). *Monogr. Rockefeller Inst. med. Res.* no. 9.
DAVIS (1933). *Biochem. Z.* **265**, 90.
— (1935). The classification and nomenclature of the lactic acid bacteria. *Proc. Soc. Agric. Bact.*
— (1936). *J. Dairy Res.* **6**, 121.
— (1937). *J. Path. Bact.* **45**, 367.
— (1939). *Nature, Lond.*, **143**, 765.
M'LEOD & GORDON (1922). *J. Path. Bact.* **25**, 139.
THOMSON & THOMSON (1927). *Ann. Pick.-Thoms. Res. Lab.* **3.**

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