THE INFLUENCE OF GROWTH MEDIUM ON THE INTERACTIONS BETWEEN *BORDETELLA PERTUSSIS* AND *STAPHYLOCOCCUS AUREUS*

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PLATE XX

MAZLOUM and ROWLEY (1955) reported that several strains of *Staphylococcus aureus* stimulated the adjacent growth of *Bordetella pertussis* on an agar medium containing charcoal. We have observed the opposite effect and report our findings herewith.

MATERIALS AND METHODS

Organisms. All organisms were obtained from departmental freeze-dried stock cultures.

Media. The following media were used: Charcoal Agar (Oxoid CM119), and charcoal agar made in our laboratory to the formulation of Oxoid Charcoal Agar from these ingredients: AR grade starch and nicotinic acid (Hopkin and Williams Ltd); AR grade sodium chloride (British Drug Houses); charcoal (undefined origin); Lab Lemco powder (Oxoid L29); Peptone (Oxoid L46); Ionagar (Oxoid no. 2). Sloppy charcoal agar and charcoal broth were also prepared in the laboratory to the same formula, except that the agar contents were respectively reduced to 0.7% or omitted.

Demonstration of inhibition. Plates of charcoal agar were flooded with a saline suspension of *B. pertussis* containing 10^4 organisms per ml. Excess suspension was removed and the inhibitory culture was streaked on or stabbed into the agar surface. Plates, enclosed in polythene bags to prevent drying, were incubated at 37°C for 5 days. Alternatively, the inhibitory colony was grown for 4 days on a sterile membrane filter placed on the agar medium. The site of growth was marked, the filter removed and the agar overlaid with 6 ml of charcoal agar which was allowed to set and then flooded with a suspension of *B. pertussis* as before. Inhibitory activity in culture filtrates was detected by placing portions of equal volume in wells cut in the agar of plates previously flooded with *B. pertussis*, and measuring the diameter of the zone of growth inhibition after incubation.

Preparation of staphylococcal culture filtrates. Cultures on sloppy charcoal agar or in charcoal broth were harvested after incubation at 37°C for 4 days; an orbital incubator was used for the broth cultures. Fluid was expressed from the sloppy agar by freezing (−20°C) and thawing (37°C) twice, and the organisms were removed from both suspensions by centrifugation. The supernatant fluids were sterilised by membrane filtration.

RESULTS

When the influence of *S. aureus* on the growth of *B. pertussis* was examined on Oxoid Charcoal Agar, a comparatively small zone of inhibition was observed (fig. 1). However, a zone approximately two-and-a-half times as large was obtained when our own charcoal agar was used (fig. 2). An unusual feature of staphylococcal colonies grown on the latter medium was their deep brown colour. When the experiment was repeated on the medium of Mazloum and Rowley (1955), stimulation of the growth of *B. pertussis* was obtained (fig. 3).

Received 20 June 1975; revised version accepted 25 Oct. 1975.

TABLE

The inhibition of growth of Bordetella pertussis strain no. 1613 by organisms other than Staphylococcus aureus

Definite inhibition	Slight inhibition	No inhibition
<i>Staphylococcus albus</i> *	<i>S. pneumoniae</i> (mucoid)	<i>Micrococcus luteus</i>
<i>Corynebacterium diphtheriae</i> var. <i>mitis</i> *	<i>B. subtilis</i>	<i>M. roseus</i>
<i>C. diphtheriae</i> var. <i>intermedius</i> *	<i>B. anthracis</i>	<i>Serratia marcescens</i>
<i>C. diphtheriae</i> var. <i>gravis</i> *	<i>Listeria monocytogenes</i>	<i>Chromobacterium violaceum</i>
<i>C. xerosis</i> *		<i>S. pyogenes</i>
<i>Streptococcus pneumoniae</i> *		<i>Klebsiella pneumoniae</i>
<i>S. faecalis</i> (haemolytic)*		<i>K. aerogenes</i>
<i>S. faecalis</i> (non-haemolytic)*		<i>Neisseria</i> (unspecified)
<i>S. viridans</i> *		<i>B. cereus</i>
<i>Escherichia coli</i> (Lilly)*		<i>B. megaterium</i>
<i>Bacillus laterosporus</i> *		<i>B. thuringiensis</i> var. <i>finitimus</i>
<i>Proteus morganii</i> *		<i>B. thuringiensis</i> var. <i>berliner</i>
<i>P. rettgeri</i> *		<i>P. inconstans</i>
<i>Shigella sonnei</i> *		
<i>Salmonella paratyphi</i> B*		

* Deep brown colouration of the colonies of these organisms on laboratory-prepared charcoal agar (see text).

The growth of 17 strains of *B. pertussis*, including NCTC no. 8189, was inhibited by *S. aureus* strain no. 41, and 17 strains of *S. aureus*, including NCTC nos. 8345 and 6571 (Oxford) inhibited the growth of *B. pertussis* no. 1613. Only one strain of *S. aureus*, no. 137B, was non-inhibitory. The inhibitory staphylococci had no common pattern of drug resistance or phage sensitivity. Several other organisms (table) also inhibited the growth of *B. pertussis*, and all the inhibitory organisms developed a deep brown colouration on laboratory-prepared charcoal agar.

When staphylococci were grown in charcoal broth, the culture filtrates showed weak inhibitory activity against the growth of *B. pertussis*, but much stronger activity was demonstrable in filtrates of cultures grown on sloppy charcoal agar. The pH values of the sloppy-agar-culture filtrates were between 4.5 and 5.0 but those of broth cultures were c. 5.3. The non-inhibitory *S. aureus* no. 137B always yielded a culture filtrate with a pH between 7.8 and 8.5. Neutralisation of the acidity in culture filtrates destroyed the inhibitory activity. The pH range for growth of *B. pertussis* was determined by adjusting the pH of different charcoal broths with sterile M sodium hydroxide or hydrochloric acid and found to lie between 6.0 and 9.0. The inhibitory factor disappeared during dialysis against phosphate-buffered saline and was therefore presumed to consist of small molecules.

DISCUSSION

The observation that 17 of 18 strains of *S. aureus* inhibited the growth of *B. pertussis* contrasts with that of Mazloum and Rowley (1955), who found that stimulation of growth occurred with 13 of 39 strains of *S. aureus*. The inhibition observed in this work was probably due to acid production, because the pH of staphylococcal culture filtrates was sometimes as low as 4.5, and neutralisation of the acidity inactivated the inhibitory property. A slightly acidic medium (pH 6.9–7.0) was recommended for the growth of *B. pertussis* by Rowatt (1957), but she also noted that early workers had reported isolating this organism on medium at pH 4.5. However, this observation was not confirmed subsequently and is in contrast to our finding that the pH range of growth, at least of *B. pertussis* no. 1613, lies between 6.0 and 9.0.

The distinctive feature of the colonies of all the organisms that inhibited the growth of *B. pertussis* was their deep brown colour on laboratory-prepared charcoal agar (table). On nutrient agar, blood agar and Oxoid Charcoal Agar the colonies were white. The pig-

ment, which was also present in inhibitory culture filtrate, was not the cause of the observed inhibition of growth of *B. pertussis* because—unlike the inhibitory activity—it was removed by dialysis.

Deletion experiments in which individual components were omitted from laboratory-prepared charcoal agar, and supplementation experiments in which components of laboratory-prepared charcoal agar were added to Oxoid Charcoal Agar, showed that starch and blood were necessary for the brown colouration of the staphylococcal colonies and for acid production. As we later observed that both of these properties could be demonstrated on blood agar containing glucose 1% (w/v), this compound may have been derived from the starch used in the laboratory-prepared medium. Glucose may be metabolised by the staphylococci to a variety of compounds, including lactic and acetic acids (Blumenthal, 1972), and the brown colour of the colonies may be due to uptake of pigments derived from the acidification of the blood in the medium. The reason why these effects were more pronounced on laboratory-prepared charcoal agar than on the Oxoid medium is not known. A comparison of free glucose levels in the two types of agar by the neocupronine method (Technicon Instruments Corporation, 1970; detection limit 50 mg per litre) indicated generally larger amounts in the laboratory-prepared medium, although results were very variable. Free glucose was not detectable in the starch used to prepare the laboratory medium but may have arisen from hydrolysis during autoclaving or from the action of amylase in the blood. However, we do not know why free glucose would not be formed in Oxoid medium under the same conditions.

It is likely that the other organisms that inhibited the growth of *B. pertussis* (table) acted in a similar manner, because their colonies also had a brown colour on laboratory-prepared charcoal medium. However, with the exception of the two micrococci and possibly the unspecified *Neisseria*, all the organisms listed in the table—whether or not inhibitory—were able to produce acid from glucose (Cowan and Steel, 1965). Thus, it seems likely that the degree of inhibition of growth of *B. pertussis* and of brown colouration of the inhibiting colonies were due to the amount or type of acid produced. However, the production of inhibitors active only in acid conditions cannot be ruled out; if produced, however, these are certainly not proteins.

When similar experiments were made with Cohen and Wheeler's (1946) medium, as used by Mazloun and Rowley (1955), stimulation of the growth of *B. pertussis*, as found by these latter workers, was observed with staphylococcal strains 41 and 137B (fig. 3). This stimulation was thought by Mazloun and Rowley (1955), and by Rowatt (1957), to be due to staphylococcal catalase which breaks down inhibitory hydrogen peroxide in the medium. As Cohen and Wheeler's medium contains starch at a concentration of 1.5 g per litre and no blood, little acid production by staphylococci is possible; indeed, we have found that the pH of liquid medium remained stable at 7.0 after seeding with *S. aureus* no. 41 and incubating for 4 days. Under these conditions the effect of catalase was significant. However, when the amount of starch in Cohen and Wheeler's medium was increased to 10 g per litre (as in charcoal agar) and 2% (v/v) of blood was added, the pH of liquid medium fell to 4.7 after seeding with the same staphylococcus and incubating for a similar time. Also, with the corresponding solid medium, inhibition of the growth of *B. pertussis* equivalent to that on charcoal agar was obtained. Thus, the growth-stimulatory effects of catalase were overcome by the large increase in acidity.

Because of the likelihood of acid production by organisms grown on media containing starch and blood, e.g., charcoal agar and Bordet-Gengou agar, we suggest that these should not be used in studies of bacterial interference. A similar caution has been put forward by Bhaskaran *et al.* (1974), who found that the growth of enteric organisms on media containing lactate, acetate or citrate might lead to the production of alkali, which mimicked the action of bacteriocines.

SUMMARY

Previous observers showed that many strains of *Staphylococcus aureus* stimulated the growth of *Bordetella pertussis* but we have found the reverse: the growth of all available

strains of *B. pertussis* on charcoal-agar medium was inhibited by a standard strain of *S. aureus*; and 17 of 18 strains of *S. aureus* (as well as several other organisms) inhibited the growth of a standard strain of *B. pertussis*. All inhibiting colonies had an unusual brown colouration on the charcoal agar used in the investigation. Both the brown colouration and the inhibitory property were caused by acid production, probably from the starch in the medium. We therefore suggest that media containing starch and blood should not be used in studies of bacterial interference.

We thank Miss M. Armstrong for some of the initial work and Professor K. B. Fraser, Dr T. Wilson (the Bacteriology Laboratory, Belfast City Hospital) and Dr J. Dunbar for advice in the preparation of the manuscript.

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EFFECT OF *S. AUREUS* ON GROWTH OF *B. PERTUSSIS*

FIGS. 1-3.—The effect of *Staphylococcus aureus* on the growth of *Bordetella pertussis* on: (1) Oxoid Charcoal Agar (top); inhibition by strain 41 and no inhibition by strain 137B; (2) laboratory-prepared charcoal agar (centre); increased zone of inhibition by strain 41 and no inhibition by strain 137B; and (3) Cohen and Wheeler's (1946) medium, as used by Mazloun and Rowley (1955) (bottom); stimulation by both strains 41 and 137B.