STUDIES ON ANTAGONISM BETWEEN HUMAN SKIN BACTERIA

P. D. MARSH* AND S. SELWYN

Department of Bacteriology, Westminster Medical School, London SW1P 2AR

PLATE X

BECAUSE mixed cultures are the rule in natural environments and pure cultures are the exception, we have become increasingly interested in interactions among bacteria, notably those that occur on the human skin. Selwyn and Ellis (1972) examined primary skin cultures from 1,320 individuals and found 173 strains belonging to the Micrococcaceae that possessed antibacterial activity. Detailed investigations have subsequently been carried out on a particularly active and stable strain of *Staphylococcus epidermidis* of biotype 4 isolated from one of us. This organism has a relatively broad spectrum of antibacterial activity due to the production of a polypeptide antibiotic of low molecular weight (Selwyn, Marsh and Sethna, 1976).

In the present paper, the results of growing the antibiotic-producing staphylococcus in mixed culture with other bacteria are compared with those obtained with a similar strain of staphylococcus that did not produce antibiotic. The studies were performed semi-quantitatively on solid media and quantitatively in liquid media.

MATERIALS AND METHODS

Bacterial strains. The antibiotic-producing strain of *Staph. epidermidis* classified as *Staphylococcus* biotype 4 (Baird Parker, 1974) and referred to as S6⁺ was isolated from the forehead of one of us (S. S.). The non-antibiotic-producing strain of *Staph. epidermidis* biotype 4 (S6⁻) was isolated from the same area. All other strains were isolated from recent clinical specimens, except for *Corynebacterium diphtheriae* var. *gravis* which was a stock culture. The micrococcus strain M7 was classified as *Micrococcus* biotype 7 (Baird-Parker, 1965) and the group-D diphtheroid according to Evans (1968). All other strains were classified by the system of Cowan and Steel (1965), and are shown in table I.

Semi-quantitative studies. The following two methods were used for studying bacterial antagonism semiquantitatively on solid medium. (1) *Direct antagonism.* Approximately 10⁶ colony-forming units (c.f.u.) of strain S6⁺ were spot-inoculated on to the surface of a plate seeded with approximately 10⁶ c.f.u. of a background strain, and both organisms were allowed to grow simultaneously for 18 h. (2) *Deferred antagonism.* Strain S6⁺ was inoculated as a line across a plate and incubated for 24 h. Bacterial growth was removed, and the remaining bacteria were killed by exposure to chloroform vapour. The test strains were inoculated at

Received 28 June 1976; accepted 31 Aug. 1976

* Present address: Medical Research Council Dental Epidemiology Unit, The London Hospital Medical College, London E1 2AD.

TABLE I

Direct and deferred antagonism between Staphylococcus S6⁺ and other bacteria

Background organism	Medium	Radii of inhibition zones (mm)	
		Direct	Deferred
<i>Staphylococcus epidermidis</i> , strain S6 ⁻	NA	0	0
<i>Micrococcus</i> sp., strain M7	NA	7	25
<i>Staph. aureus</i>	NA	5	21
<i>Staph. aureus</i> *	NA	3	18
<i>Streptococcus pyogenes</i>	BA	5	20
Viridans streptococcus	BA	4	...
<i>Strep. pneumoniae</i> type 3	BA	4	...
Faecal streptococcus	BA	1	...
<i>Clostridium welchii</i>	BA	3	...
Group-D diphtheroid	NA	10	33
<i>Corynebacterium diphtheriae</i> var. <i>gravis</i>	NA	3	...
<i>C. diphtheriae</i> var. <i>intermedius</i>	NA	4	20
<i>Neisseria catarrhalis</i>	NA	2	...
<i>N. gonorrhoeae</i>	CA	0	24
<i>Pasteurella septica</i>	NA	4	...
<i>Haemophilus influenzae</i>	CA	0	17
<i>Proteus mirabilis</i>	NA	0	0
<i>Pseudomonas aeruginosa</i>	NA	0	0
<i>Escherichia coli</i>	NA	0	...
<i>Klebsiella pneumoniae</i>	NA	0	...

NA = nutrient agar; BA = blood agar; CA = chocolate agar.

... = not tested.

* This strain was resistant to penicillin, tetracycline, erythromycin, methicillin, gentamicin, fusidic acid, lincomycin and chloramphenicol.

right angles to the line of original growth and the plate was reincubated for 18 h. In both methods, zones of inhibition were recorded as shown in table I.

Replicate plates were made of cultures with inhibition zones to assess the extent of bactericidal and bacteristatic activity. The timing of antibiotic production was determined by removing the growth of the "producer" organism after varying intervals in the deferred test, and by using time-lapse photomicrography to study the development of inhibition zones in direct antagonism tests (Marsh, 1975). Antibiotic production by strain S6⁺ was detected against suitable indicator strains under a variety of conditions aerobically and anaerobically and on different media including nutrient agar with NaCl 5% w/v, Tween 80 0.5% w/v, or oleic acid 1.0% v/v. All the standard media listed in tables I and II were prepared as described by Cruickshank (1965).

Quantitative studies. Antagonism was investigated quantitatively by the use of pure and mixed liquid batch-cultures. Viable counts were determined on selective media by the method of Miles, Misra and Irwin (1938). The combinations of bacteria with their appropriate growth and selective media are shown in table II. Glucose broth consisted of nutrient broth with the addition of glucose 0.5% w/v; serum broth consisted of nutrient broth with the addition of horse serum 10% v/v; serum broth plus catalase consisted of serum broth with the addition of 100 enzyme units per ml of catalase (British Drug Houses, Ltd) in 0.2M phosphate buffer at pH 7.0. Selective media were developed according to the antibiotic-sensitivity patterns and MICs of particular antibiotics for the relevant bacteria (Marsh, 1975).

Batch cultures were incubated aerobically at 33°C in 150-ml conical flasks stirred magnetically. The appropriate culture fluids in 20 ml quantities were inoculated with 0.1 ml of various dilutions of overnight cultures of the required bacterial strains. Viable counts were

TABLE II
Viable counts in mixed batch cultures

Mixed cultures (strain number or designation)	Growth medium	Selective medium	Log. viable count (c.f.u. per ml) at	
			12 h	30 h
S6 ⁺ S6 ⁻	NB	NA+FA	8.7	8.6
		NA+P	7.2	8.3
S6 ⁺ M7	NB	NA+B	6.4	8.2
		NA+F	6.3	5.6
S6 ⁻ M7	NB	NA+B	7.2	7.9
		NA+F	7.1	8.0
S6 ⁺ Group-D diphtheroid	NB	NA+B	6.6	8.5
		NA+F	3.6	0
S6 ⁻ Group-D diphtheroid	NB	NA+P	8.3	8.6
		NA+F	5.4	6.0
S6 ⁺ <i>C. diphtheriae</i> var <i>gravis</i>	NB	NA+B	5.0	7.6
		Tellurite	5.1	7.1
S6 ⁺ Viridans streptococcus	GB	MacConkey	8.5	8.8
		BA+CV	7.5	8.4
S6 ⁺ Faecal streptococcus	GB	NA+F	9.3	8.9
		BA+CV	9.3	8.8
S6 ⁺ <i>Streptococcus pneumoniae</i>	SB	MacConkey	4.0	0
		BA+CV	8.3	6.3
S6 ⁻ <i>Strep. pneumoniae</i>	SB	NA+P	6.0	0
		BA+CV	8.8	6.5
S6 ⁺ <i>Strep. pneumoniae</i> (inoculated after 4 h)	SB	MacConkey	7.5	7.9
		BA+CV	8.1	8.3
S6 ⁻ <i>Strep. pneumoniae</i> (inoculated after 4 h)	SB	NA+P	7.5	7.5
		BA+CV	8.2	8.5
S6 ⁺ <i>Strep. pneumoniae</i>	SB+catalase	MacConkey	7.0	7.3
		BA+CV	7.6	7.3
S6 ⁻ <i>Strep. pneumoniae</i>	SB+catalase	NA+P	6.3	6.5
		BA+CV	8.2	8.1
S6 ⁺ <i>Strep. pyogenes</i>	SB	MacConkey	8.7	7.9
		BA+CV	7.0	6.9
S6 ⁻ <i>Strep. pyogenes</i>	SB	MacConkey	6.1	4.4
		BA+CV	8.7	8.0
S6 ⁺ <i>N. catarrhalis</i>	GB	NA+B	7.3	6.9
		NA+T	7.9	8.0
S6 ⁻ <i>N. catarrhalis</i>	GB	NA+P	6.8	5.8
		NA+T	8.2	7.1
<i>Staphylococcus aureus</i> with S6 ⁺	NB	PPA	8.4	7.3
with S6 ⁻			7.4	7.1
with M7			6.8	6.3
with group-D diphtheroid			5.8	6.3
			5.1	5.6

c.f.u. = Colony-forming units.

NB = Nutrient broth; GB = glucose broth; SB = serum broth; SB+catalase = serum broth+100 units of catalase per ml in 0.2M phosphate buffer, pH 7.0; NA+FA = nutrient agar+fusidic acid 0.196 µg per ml; NA+P = nutrient agar+penicillin 0.1 µg per ml; NA+B = nutrient agar+bacitracin 0.1 unit per ml; NA+F = nutrient agar+furazolidone 7.5 µg per ml; NA+T = nutrient agar+trimethoprim 15.0 µg per ml; BA+CV = blood agar+crystal violet 1 in 666 666 (w/v); PPA = phenolphthalein-phosphate agar.

taken after 0, 4, 8, 12 and 30 h and growth curves were constructed. Strains were grown initially in pure cultures, and then under similar conditions in mixed cultures.

The timing of antibiotic production in liquid medium was determined by growing pure cultures of strain S6⁺ in nutrient broth. Culture samples of 1 ml were taken at hourly intervals, bacteria were removed by centrifugation and Millipore filtration (average pore diameter 0.45 μ m), and the supernate was spotted on to plates seeded with a sensitive indicator organism (M7). After incubation, zones of inhibition were measured.

RESULTS

Semi-quantitative studies

In direct antagonism tests, strain S6⁺ was active against all gram-positive strains tested—with the notable exception of strain S6⁻, which had been derived originally from the same skin area. There was, however, only limited activity against gram-negative bacteria (table I). In a control experiment, strain S6⁻ had no antibacterial activity against any of the test strains. In deferred antagonism tests, strain S6⁺ had a broader spectrum of activity and also produced larger zones than in direct antagonism procedures. Replicate plating of inhibition zones produced by strain S6⁺ indicated that the antibiotic has a bactericidal mode of action. No colonies of the test strains grew in the replicated inhibition zones, except at the periphery.

Direct antagonism viewed under a plate microscope was evident within 2 h of culture and was well developed by 4 h (fig. 1*a*). Subsequent growth by the background test strain served only to emphasise this zone of inhibition (fig. 1*b*). Deferred antagonism techniques detected inhibition zones after 7 hours' growth of the producer S6⁺ strain. After this threshold period of growth, zone sizes were larger with this method than with direct antagonism. Inhibition zones were detected under aerobic and anaerobic conditions and in the presence of NaCl 5%, Tween 80 0.5% and oleic acid 1.0%.

Quantitative Studies

Viable counts of the different mixed cultures at 12 and 30 h are shown in table II. Mixed batch cultures of strains S6⁺ and S6⁻ did not demonstrate any mutual or unilateral antagonism. Both strains grew as well together in mixed culture as they did in pure culture. Strain S6⁻ did not inhibit the growth of any other strain in batch culture. The only detectable difference between strains S6⁺ and S6⁻ was the ability of the former to produce an antibiotic.

With strain S6⁻, growth of strain M7 reached virtually the same level as when it was grown in pure culture. In contrast, unilateral antagonism of the strain M7 occurred within 11 h when it was grown in mixed culture with strain S6⁺. After 28 h, the growth of strain M7 was 300-fold less than in pure culture and 200-fold less than in mixed culture with strain S6⁻.

The viable counts of the group-D diphtheroid in mixed culture with strain S6⁻ were lower than in pure culture, but the diphtheroid remained capable of division and even after incubation for 56 h it was still slowly increasing in

FIG. 2.—Growth curves of mixed batch cultures of (a) strain S6⁺ and group-D diphtheroid (D), (b) strain S6⁻ and group-D diphtheroid (D), in nutrient broth at 33°C.

FIG. 3.—Growth curves of mixed batch cultures of (a) strain S6⁺ and *Streptococcus pneumoniae* (PN), (b) strain S6⁻ and *Strep. pneumoniae* (PN), in serum broth plus 100 units per ml of catalase (in 0.2M phosphate buffer, pH 7.0) at 33°C.

FIG. 4.—Growth curves of mixed batch cultures of (a) strain S6⁺ and *Strep. pyogenes* (SP), (b) strain S6⁻ and *Strep. pyogenes* (SP) in serum broth at 33°C.

viable count (fig. 2*b*). However, in mixed culture with strain S6⁺ the viable count of the group-D diphtheroid fell below the detectable level of the counting method after 16 h (fig. 2*a*). The diphtheroid was still not detected when whole plates of selective media were flooded with culture fluid. In liquid medium, strain S6⁺ did not inhibit the growth of *C. diphtheriae* var. *gravis*.

In separate experiments, strain S6⁺ was grown with two strains of viridans streptococci with different results. In one mixed culture, the two strains grew together without any interaction; in the other, strain S6⁺ was markedly inhibited over the final 6 h of growth. Similarly, when strain S6⁺ was grown with two strains of faecal streptococci in separate experiments, two different results were obtained. No interaction was detected with one strain, but with the other the viable counts of strain S6⁺ after 29 h were 100-fold lower than in pure culture.

In serum broth, *Strep. pneumoniae* type 3 was partially inhibited by strains S6⁺ and S6⁻, whereas the two staphylococci were completely inhibited by the pneumococcus in mixed culture in glucose broth. Although the pH fell, it did not reach inhibitory levels. When the experiment was repeated in serum broth, but with the pneumococcus inoculated after 4 h, both strains S6⁺ and S6⁻ were able to grow to levels approaching those in pure culture. When serum broth supplemented with catalase was used as the growth medium for the same mixed cultures, strain S6⁺ was unaffected by the pneumococcus and was able to inhibit the latter eight-fold in comparison with its growth in pure culture (fig. 3*a*). However, strain S6⁻ was still inhibited five-fold (fig. 3*b*). Figs. 4*a* and 4*b* show the mixed-culture results of strain S6⁺ and strain S6⁻ with *Strep. pyogenes*. Strain S6⁺ inhibited the streptococcus almost 50-fold whereas the streptococcus inhibited strain S6⁻ 500-fold in comparison with its growth in pure culture. In mixed culture with *Neisseria catarrhalis*, both strains S6⁺ and S6⁻ showed no antagonistic effect but instead there was a slight reduction in the growth of the staphylococci.

The results of mixed cultures of a non-antibiotic-producing strain of *Staph. aureus* with the skin commensals were difficult to analyse because a suitable selective medium for the differential counts was not discovered. The M7 and group-D diphtheroid strains were partially inhibited by *Staph. aureus*, though both strains were still slowly increasing in viable count after 30 h. Strains S6⁺ and S6⁻ each grew without marked inhibition when mixed with *Staph. aureus*, though strain S6⁻ did not reach the population level attained by strain S6⁺.

Antibiotic production by strain S6⁺ in liquid medium was detected after incubation for 13 h at 33°C.

DISCUSSION

The quantitative results from batch cultures confirmed the semi-quantitative findings on solid media that strain S6⁺ had no effect on strain S6⁻, and that strain S6⁻ did not significantly inhibit any other strain. The only known difference between the two organisms was the ability of strain S6⁺ to produce

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FIG. 1a.—Direct antagonism; inhibition zone after incubation for $3\frac{1}{2}$ h; strain S6⁺ stab at left, initial background growth of *Staphylococcus aureus* just visible at right. $\times 12$.

FIG. 1b.—The same field as in fig 1a after incubation for $12\frac{1}{2}$ h. $\times 12$.

an antibiotic. Thus, in the mixed batch-cultures strain S6⁻ acted as a control and any effect in a mixed culture with S6⁺ not observed in one with S6⁻ was assumed to be due to antibiotic.

Unilateral antagonism by strain S6⁺ was demonstrated in mixed batch cultures against strain M7, the group-D diphtheroid and *Strep. pyogenes*. In similar mixed cultures, strain S6⁻ had no effect against strain M7, the growth of the diphtheroid was only slightly slowed by strain S6⁻, and strain S6⁻ was itself markedly inhibited by *Strep. pyogenes*. Thus antibiotic production conferred an advantage on strain S6⁺ in these mixed cultures.

Over 50 years ago, McLeod and Gordon (1922) showed that hydrogen-peroxide production by a pneumococcus caused its auto-inhibition. This observation was confirmed and extended in the present study of mutual antagonism between a pneumococcus and strain S6⁺ in catalase-supplemented broth. Although strain S6⁻ was still slightly inhibited, strain S6⁺ under the same conditions was able to inhibit the pneumococcus. Similarly, adequate initial catalase production by the staphylococci themselves probably prevented their subsequent inhibition when they were inoculated 4 h before the pneumococcus, and also prevented auto-inhibition of the pneumococcus in the resulting mixtures.

No antagonism could be detected between strain S6⁺ and *Staph. aureus* in liquid medium, whereas on solid media strain S6⁺ powerfully inhibited strains of *Staph. aureus* (including multiple antibiotic-resistant strains). Discordant results in the two systems were also obtained against *C. diphtheriae*, and the paradoxical inhibition of strain S6⁺ in liquid media by some viridans and faecal streptococci, pneumococci and *N. catarrhalis* were presumably due to dilution of the S6⁺ antibiotic to below the bacteristatic level while inhibitory products of the other organisms remained at active concentrations. The effect of dilution was probably also responsible for the fact that there was a longer interval before both unilateral antagonism and antibiotic production could be demonstrated in liquid than on solid media. In the latter, the polypeptide antibiotic diffuses to a certain extent, but concentrations in the neighbourhood of producer colonies are relatively high.

Similarly, the failure to demonstrate in liquid media a uniformly bactericidal effect for the S6⁺ antibiotic—except against the extremely sensitive group-D diphtheroid—can be ascribed to dilution. Thus, the high local concentrations in agar can readily reach bactericidal levels, whereas the titres in broth produced by relatively fewer cells dispersed in a large volume of fluid appear usually to attain only bacteristatic levels. Simple tests for antagonism on solid media are therefore sensitive screening procedures before more detailed studies in liquid cultures are undertaken. The latter, however, not only provide quantitative data but also permit more than two organisms to be studied; and they allow insight into the kinetics of inhibition. A longer term study of these interactions has been made in a simple continuous-culture system (Marsh and Selwyn, 1977).

The antibiotic from strain S6⁺ was produced under aerobic and anaerobic conditions and in the presence of both 5% sodium chloride and unsaturated fatty acids. The latter finding contrasts with the narrower spectrum antibiotic

activity of *Staph. aureus* type 71 studied by Barrow (1963). Thus the S6⁺ antibiotic is produced under conditions found on the skin surface and has its greatest activity against the types of bacteria most likely to be encountered there. Production of the antibiotic during the logarithmic growth phase is in contrast with the production of the chemically analogous antibiotics by species of *Bacillus*, which occurs in the stationary growth phase and is believed to be a factor in repressing vegetative cell functions and promoting sporulation (Sarkar and Paulus, 1972).

The competitive advantage that antibiotic production has been shown to confer on strain S6⁺ provides a basis for the results of a recent epidemiological study on the protective effect of similar organisms in skin lesions (Selwyn, 1975). Although strain S6⁺ itself is antagonistic to few gram-negative bacteria, its semi-purified antibiotic has a surprisingly wide antimicrobial spectrum, including action on *Pseudomonas* spp., and even *Candida* spp. (Selwyn *et al.*, 1976). The significance of this finding in the natural defence of the skin against colonisation by pathogens has yet to be determined. Certainly the possible accumulation of such antibiotics in the epidermis should be investigated, particularly in skin lesions containing large bacterial populations.

SUMMARY

Antagonism by an antibiotic-producing *Staphylococcus epidermidis* strain (S6⁺) against other bacteria was studied semi-quantitatively on solid media and quantitatively in liquid batch culture. Parallel experiments were performed with a similar but non-antibiotic producing strain (S6⁻). On solid media strain S6⁺ in the logarithmic growth phase unilaterally inhibited members of all gram-positive species tested, whereas S6⁻ had no effect. In liquid media, more complex interactions were observed, including mutual and reversed antagonisms. The relevance of these findings to the ecology of human skin is discussed.

P. D. M. was initially supported by a grant from Ethicon Limited and subsequently by one from Beecham Products Limited.

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