CHARACTERISTICS OF A STRAIN OF *STAPHYLOCOCCUS AUREUS* GROWN IN VIVO AND IN VITRO

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ABSTRACT

BEINING, PAUL R. (The Catholic University of America, Washington, D.C.) AND E. R. KENNEDY. Characteristics of a strain of *Staphylococcus aureus* grown in vivo and in vitro. J. Bacteriol. **85**:732–741. 1963.—A comparative survey was conducted on the characteristics of a strain of *Staphylococcus aureus* after it had been grown in vitro (VSB) and after it had been collected from the peritoneal exudate of an infected guinea pig (GSB). Both VSB and GSB strains gave the same results when studied in an extensive series of tests, including bound and soluble coagulases, bacteriophage type, antibiotic-sensitivity pattern, the usual fermentation reactions, deoxyribonucleic acid base composition, and qualitative tests for hemolysins, deoxyribonuclease, ribonuclease, staphylokinase, staphylo-protease, lipase, and phosphatase. The in vivo strain differed significantly from the in vitro strain in respiratory rate, agar gel diffusion studies, agglutinability in tube tests, virulence tests in rabbits and mice, growth on tellurite-glycine agar, susceptibility to human γ-globulin in agar, and in the quantitative production of deoxyribonuclease, α-hemolysin, leucocidin, and hyaluronidase.

Since the work of Van de Velde (1894), the notion has persisted that virulence can be increased by animal passage and is often progressively lost during artificial cultivation. The impressive array of facts on the biochemical, serological, and virulence properties of *Staphylococcus aureus*, amassed from in vitro studies, may not be reflecting the characteristics of true in vivo staphylococci.

The study of bacteria recovered from an in vivo environment has been impeded by the technical difficulty of collecting large quantities of bacteria free from extraneous material. Smith, Keppie, and Stanley (1953) established a workable method for collecting and separating large amounts of bacteria from the tissue fluid of infected guinea pigs. With this procedure, Gellenbeck (1962) showed that her in vitro strain of *S. aureus* had a significantly lower exogenous respiratory rate than did the same strain after it had passed through a guinea pig.

The purpose of the present investigation was to conduct a comparative survey of the characteristics of a strain of *S. aureus* after it had been grown in vitro (VSB) and after it had been collected from the peritoneal exudate of an infected guinea pig (GSB). The more pertinent biochemical, serological, and virulence tests were employed in the survey.

MATERIALS AND METHODS

Source, preparation of suspensions, and maintenance of S. aureus grown in vitro. The *S. aureus* was obtained from Providence Hospital, Washington, D.C., and represented a culture from a single colony taken from a sheep-blood agar plate that had been streaked with pus from a fresh furuncle. Several slants, representing the first passage after isolation, were made, stored at 5 C, and used as stock cultures for all further preparations. The staphylococci were cultured on Trypticase Soy Agar (BBL) slants or in 10 ml of Trypticase Soy Broth and incubated aerobically for 18 hr at 37 C. The in vitro supernatant fluid was prepared by growing the organisms in broth culture for 18 hr; the cells were removed by centrifugation of the culture in the cold (2 C) at 10,000 $\times$ *g* for 30 min. The sedimented bacteria were suspended with a glass rod in a small amount of 0.1 M Veronal-buffered saline (pH 7.3); these bacteria were called the in vitro-suspended cells (VSB cells). Cells and supernatant were stored at 5 C.

Inoculation and recovery of S. aureus from guinea pigs. The inoculum for the injection of guinea pigs was prepared by harvesting 24-hr

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cultures of VSB cells from five Trypticase Soy Agar slants and suspending the cells in 10 ml of buffer. This suspension represented approximately 6×10^9 viable cells per ml. A male guinea pig was injected, and the organisms recovered and purified as reported previously (Gellenbeck, 1962).

The guinea pig peritoneal supernatant fluid was removed and immediately stored in 2-ml amounts at −20 C. The cells recovered from the animal's body fluids were designated the GSB cells, and were stored at 5 C.

Biochemical and metabolic studies. Aerobic respiratory rates of VSB and GSB cells in 0.0125 M glucose and in buffer (endogenous) were determined by the procedure of Gellenbeck (1962).

Minimal staphylococcal agar was made according to the method of Burns and Holtman (*personal communication*) and contained (mg per 100 ml): glucose, 100; Difco vitamin-free Casamino Acids, 500; thiamine HCl, 0.5; and agar, 1,500.

The γ-globulin was poliomyelitis-immune (human) globulin, purchased from Merck Sharp & Dohme, Philadelphia, Pa. Each ml of this material contained 0.16 g of globulin and represented a pooling of material from 2,000 donors. Sterile filter-paper strips (15 by 60 cm) were soaked in the γ-globulin and placed on medium identical with that used by Anderson (1956). Plates were incubated for 48 hr in an atmosphere of 30% CO_2 in an anaerobic incubator (National Appliance Co., Portland, Ore.).

Fibrinolysis of heat-precipitated human fibrin by staphylokinase or by protease, when soybean trypsin inhibitor (Nutritional Biochemicals Corp., Cleveland, Ohio) was added to the medium, was detected by the procedure of Lack and Wailling (1954).

Titration of hyaluronidase and deoxyribonuclease activity was performed by the ACRA test, according to the modification of Oakley and Warrack (1951).

Preparation of antisera. Female albino rabbits (2 to 3 kg) were used for production of antisera. For sera against whole bacterial cells, suspensions of live VSB and GSB cells and GSB cells killed with Zephiran (Winthrop Laboratories, New York, N.Y.), according to the procedure of McCoy and Kennedy (1960), were injected intravenously over a 16-day period. The period consisted of two series of six daily injections with a 4-day rest separating the two series. A total of approximately 6.8×10^8 cells was given in the 16-day period. Antisera were prepared from blood taken by cardiac puncture 5 days after the last injection and stored, without preservative, at −20 C. For sera against soluble staphylococcal products, alum-precipitated suspensions of in vitro and in vivo supernatant fluids were used and injected by the intravenous route. Three injections per week were given over a 6-week period. Antisera were collected 5 days after the last injection and stored at −20 C.

To prepare a control rabbit antiserum, a guinea pig was injected with *Escherichia coli*; its in vivo supernatant was collected in the same manner as described previously for staphylococci. The control rabbit antiserum was prepared against this in vivo supernatant fluid of *E. coli* in the same manner as described above for the GSB supernatant.

Virulence studies: pathogenicity of in vivo and in vitro cells. VSB and GSB organisms in buffer (at a concentration of approximately 10^8 cells per ml) were serially diluted, and 1 ml of the dilutions was injected intraperitoneally into five albino mice weighing 18 to 25 g. Examination of mice ended 48 hr after inoculation. Portions (0.1 ml) of the same dilutions were injected intradermally into depilated albino rabbits weighing 2 to 3 kg. The site of injection was observed for 4 days.

Pathogenicity of VSB and GSB organisms for rabbits was also determined by the intravenous route. Samples of suspensions (which originally contained approximately 7×10^7 organisms per ml) were injected according to the schedule for immunizing with particulate antigens. Injections were stopped when osteomyelitis, organ involvement, or death appeared.

Toxicity of in vivo and in vitro supernatant fluid: in mice. Portions (1 ml) of serial twofold dilutions of the supernatant fluids were injected intraperitoneally into groups of five albino mice (18 to 25 g). The highest dilution producing death in 50% of the animals within 3 days was taken as the end point of titration.

In rabbits. Portions (0.1 ml) of the same serial dilutions were injected intradermally; the first dilution failing to give a dermonecrotic reaction within 4 days was considered the end point of titration.

TABLE 1. *Respiratory response of Staphylococcus aureus strains VSB and GSB*

Substrate	Respiration (O_2 uptake)					
	At 60 min			At 120 min		
	GSB	VSB	Percentage increase	GSB	VSB	Percentage increase
	μliters	μliters		μliters	μliters	
Glucose, 0.2 M.	210	92.6	78*	113	78.4	31*
Endogenous respiration (buffer)	1.35	5.75	76†	2.6	5.4	52†

* Percentage of increase with respect to GSB.
† Percentage of increase with respect to VSB.

RESULTS

Strains VSB and GSB exhibited the size, shape, and staining properties typical of staphylococci. Both strains were catalase-positive, showed hemolysis on rabbit and human red blood cell plates only, possessed bound and soluble coagulases, and were bacteriophage type 80/81. Both strains were sensitive to chloramphenicol, oleandomycin, neomycin, nitrofurantoin, methenamine mandelate, and novobiocin; both strains were resistant to penicillin, dihydrostreptomycin, oxytetracycline, erythromycin, chlortetracycline, triple sulfa, sulfamethizole, sulfisoxazole, sulfadiazine, and sulfathiazole. The two strains produced brilliant golden-orange coloration with aerobic incubation on skim-milk agar plates; when incubated in 30% CO_2, both strains grew well but all colonies were chalk-white.

The possibility that passage through a guinea pig caused the emergence of organisms with a different deoxyribonucleic acid (DNA) base composition was tested by several thermal denaturation curves on both strains. There was a close similarity between VSB and GSB strains relative to the thermal denaturation profile (T_m) and the per cent guanine plus cytosine (GC content). The T_m of VSB was 85.3 C and GC content 39%, whereas the GSB strain showed a T_m of 85.5 C and 39.5% GC content. The slight difference in T_m between the two strains is within the bound of experimental error and the consistency of the apparatus used. The technical difficulty of obtaining enough GSB cells directly from guinea pigs for the DNA extraction procedure necessitated the passing of strain GSB once into Brain Heart Infusion broth after removal from the guinea pig.

The above characteristics, shared by the VSB and the GSB strains, were considered to be the principle markers of this culture of *S. aureus*. Frequently, through the course of this investigation, stock cultures and subcultures were tested for these markers to establish identity with the original isolate used in the study.

Biochemical and metabolic studies. As Gellenbeck (1962) demonstrated with her strain, the aerobic respiratory rate of our GSB strain was higher than that of the VSB strain, although the endogenous respiratory rate of the VSB strain was significantly higher than that of the GSB strain. This relationship is shown in Table 1.

Studies with potassium tellurite-glycine agar indicated a significant difference in the metabolic capacity of in vitro and in vivo strains. The surface of all plates was inoculated with one loopful of bacterial suspension containing approximately 10^3 organisms per ml, and readings were made 24 hr later. Under certain defined experimental conditions, the GSB strain was completely incapable of growing on potassium tellurite-glycine agar and of reducing tellurite within the 24-hr period, whereas the VSB strain was able to grow and reduce the tellurite. The ability of strain VSB, but not GSB, to grow on potassium tellurite-glycine agar (final pH of 6.3 before pouring the plate) and to reduce the tellurite is evident from Fig. 1; the right half of this figure shows that both strains grew equally well on a control Trypticase Soy Agar plate. The effect of pH on the differential selectivity of potassium tellurite-glycine agar for VSB, GSB, and *S. epidermidis* strains is recorded in Table 2. The medium at pH 5.0 was inhibitory to all strains. However, at pH 6.3, all GSB isolates and *S. epidermidis* were inhibited by the medium, while the VSB strain grew well and reduced the tellurite. When the pH was 7.2, a few of the GSB isolates began to reduce the tellurite and grow on the medium. A pH of 9.6 was also less favorable to growth of the GSB strain; but, at this pH, *S. epidermidis* began to grow and to reduce the tellurite. The majority of GSB isolates were incapable of growing and reducing tellurite over the pH range used in this study. Since it was possible that some factor in the in vivo super-

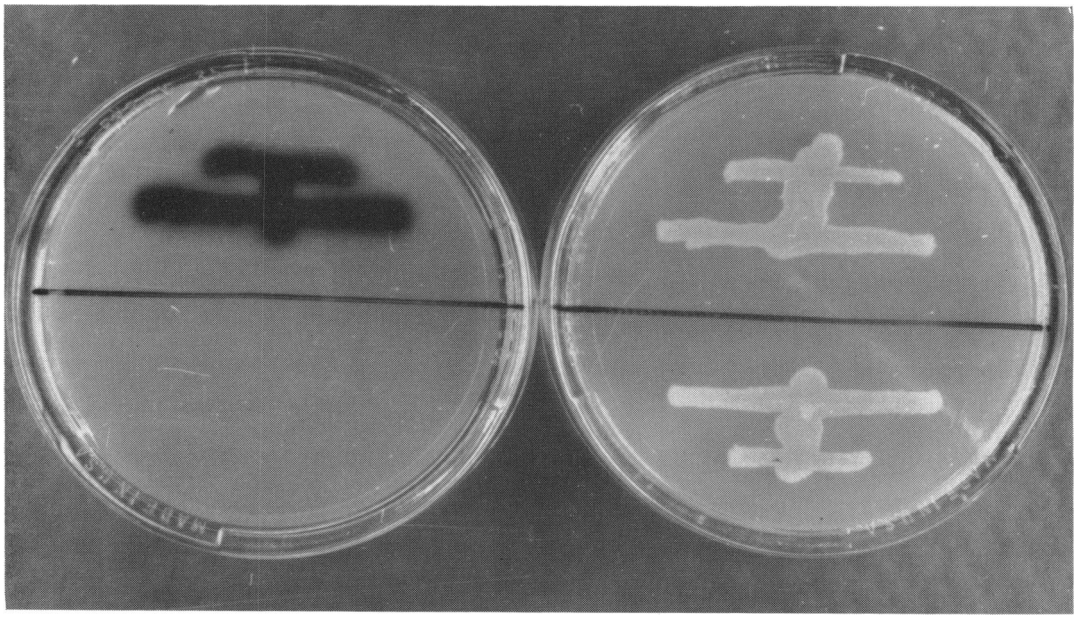

FIG. 1. *Differential growth of strains VSB and GSB within 24 hr on potassium tellurite-glycine agar (final pH 6.3). Upper half of the tellurite-glycine agar plate on the left of the figure contained the VSB strain; the lower half of that plate was streaked with the GSB strain. The Trypticase Soy Agar plate on the right of the figure was streaked in the same manner with VSB and GSB strains. All streaks were made with a loopful of organisms from a suspension in Veronal-buffered saline containing approximately 10^8 organisms per ml.*

TABLE 2. *Effect of pH on the differential selectivity of potassium tellurite-glycine agar for Staphylococcus epidermidis, VSB, and seven GSB isolates**

Substrate	pH	Bacterial strains				
		S. epidermidis	VSB	GSB isolate a	GSB isolate b	GSB isolates c to g
Tellurite-glycine agar	5.0	0	0	0	0	0
	6.3	0	4+	0	0	0
	7.2	0	4+	4+	4+	0
	9.6	2+	2+	0	2+	0
Trypticase Soy Agar		4+	4+	4+	4+	4+

* All GSB isolates represent distinct harvests from different guinea pigs. The pH values are final readings made immediately before pouring plates. The 4+ indicates as much growth on tellurite-glycine agar as seen on the Trypticase Soy Agar-plate control for that isolate; 2+ would indicate approximately one-half as much growth as the control.

natant was acting as an inhibitor and preventing strain GSB from metabolizing on this medium, a pilot experiment was set up to determine the effect of the supernatant fluid itself. Fresh GSB supernatant was incapable of reducing tellurite and could not inhibit its reduction when mixed with VSB isolates which were capable of reducing tellurite. As noted by Zebovitz, Evans, and Niven (1955), we also found that potassium tellurite from various commercial sources differed in selective activity. The nature of inhibition of growth of strain GSB in this medium is being investigated further.

Another manifestation of difference in the metabolic capacity of in vivo and in vitro strains is seen in Fig. 2. When *S. epidermidis*, VSB, and GSB were streaked at right angles to γ-globulin–impregnated filter paper, there was a differential inhibition of growth due to the γ-globulin. The concentration of γ-globulin in the filter paper, apparently, was an important factor because the same isolate (*S. epidermidis*, VSB, or GSB) was inhibited to a slightly different degree on different occasions. However, when the three strains were streaked on the same plate, the GSB strain was

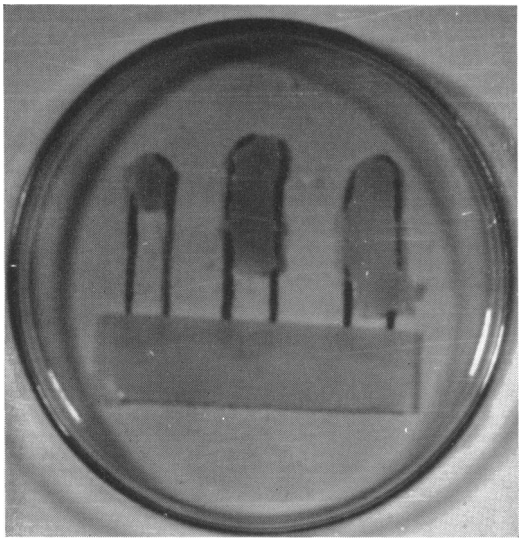

FIG. 2. *Differential inhibition of growth of strains GSB, VSB, and Staphylococcus epidermidis due to γ-globulin–impregnated filter paper in Anderson medium. From left to right, the strains are GSB, VSB, and S. epidermidis, respectively.*

always inhibited more than the VSB or *S. epidermidis* strains. When nine different isolates of the three strains were tested, the average distance of inhibition by γ-globulin was: strain GSB, 15 mm; strain VSB, 9 mm; *S. epidermidis*, 7 mm. The γ-globulin effect could not be demonstrated when filter-paper strips were impregnated with guinea pig complement (BBL), normal rabbit serum, rabbit anti-GSB serum, or with anti-α-toxin serum.

Studies on the minimal staphylococcus agar of Burns and Holtman (*personal communication*) indicated that the GSB strain is much less capable of metabolizing and growing than is the VSB strain. On the surface of minimal agar plates, 1 ml of each strain (containing 100 to 300 organisms per ml) was spread. The VSB strain grew almost as luxuriously on this medium as it did on the Trypticase Soy Agar control medium, but only five colonies of the GSB strain grew on the minimal agar as opposed to 200 colonies on the control agar. Similarly, limited experiments indicated that strain GSB grew less readily on polymyxin B and phenylethyl alcohol agar than did strain VSB; strain GSB, however, was less inhibited on tetrazolium agar (Kennedy and Barbaro, 1952) plates than was the VSB strain.

The reactions of strains VSB and GSB agreed with the description of reactions of *S. aureus* in *Bergey's Manual* (Breed, Murray, and Smith, 1957) with respect to liquefaction of gelatin, failure to produce indole, reduction of nitrates, and tube fermentation tests with lactose, raffinose, mannitol, dextrose, and sucrose. However, strain GSB did hydrolyze starch with the production of acid both aerobically and in 30% CO_2, whereas strain VSB was incapable of hydrolyzing this polysaccharide.

Studies on diffusible cellular products. Strains GSB and VSB were also compared as to their qualitative and, where possible, quantitative production of several extracellular, staphylococcal "elaborates" in vitro. Qualitatively, both the GSB and VSB strains produced catalase, bound and soluble coagulase, α-hemolysin, α-hemolysin, leucocidin, deoxyribonuclease, ribonuclease, staphylokinase, staphyloprotease, lipase, and phosphatase.

A significantly greater amount of α-hemolysin, leucocidin, hyaluronidase, and deoxyribonuclease was produced by those organisms which had been cultivated in the in vivo environment (Table 3). In conjunction with the titrations listed in Table 3, a neutralization test was performed with a *Staphylococcus* antiserum (CPP 101/63A), obtained from Wellcome Research Laboratories and known to contain 480 units of anti-α-hemolysin. The antiserum completely neutralized the α-hemolytic activity of undiluted GSB supernatant.

Serological studies. In Table 4 are listed the

TABLE 3. *Quantitative production of hemolysins, leucocidin, deoxyribonuclease, and hyaluronidase by VSB and GSB strains*

Soluble titration product	Supernatant fluid		
	GSB	VSB	*Escherichia coli* guinea pig
α-Toxin*	102.4	0	0
β-Toxin*	20	0	0
δ-Toxin*	2	0	0
Hyaluronidase†	1,280	16	2
Deoxyribonuclease†	1,024	4	32
Leucocidin‡	64	1	Not tested

* Units represent the reciprocal of the highest dilution giving 50% hemolysis.

† Units represent the reciprocal of the highest dilution to give a firm clot in the ACRA test.

‡Units represent the number of minimal leucocidal doses.

TABLE 4. *Comparison of strains GSB and VSB by the tube agglutination test*

Antiserum	Antigen	
	VSB	GSB
Preimmunization sera.	<1:4*	<1:4*
Normal rabbit serum.....	<1:4	<1:4
Normal guinea-pig serum.....	<1:4	<1:4
Anti-GSB (Zephiran-killed).....	<1:10	1:320
Anti-GSB (living).....	1:5,120	1:320†

* Figures represent the highest dilution of serum giving 2+ or higher reading.

† Thread agglutination.

TABLE 5. *Cross-precipitation pattern in Oudin tubes* of VSB, GSB, and Escherichia coli guinea-pig supernatant fluids against homologous and heterologous antisera†*

Internal reactant	External reactant (zones)		
	GSB supernatant	VSB supernatant	*E. coil* guinea-pig supernatant
Anti-GSB supernatant....	5	0	3
Anti-*E. coli* guinea pig supernatant.....	3	0	3
Normal rabbit serum.....	0	0	0

* Recording after 5 days at 25 C.

† Final dilution of serum was 1:2.

agglutination titers of strains VSB and GSB in the tube agglutination test. In addition to the difference in titer of the two strains, only the GSB cells exhibited "thready" agglutination in antisera against the living GSB organism. The results imply that a different major antigenic component(s) is present on the surface of the GSB and VSB strains; the results could also imply that killing with the detergent Zephiran destroyed a major antigen(s) more pertinent to the VSB than to the GSB strain.

Studies on the antigenic cellular "elaborates" of strains VSB and GSB were made by the Oudin technique. Table 5 gives the cross pattern of precipitation of VSB and GSB supernatant fluids against their homologous and heterologous antisera. *E. coli* guinea pig supernatant and the antiserum prepared against it were included in the Oudin tests as a control to determine the

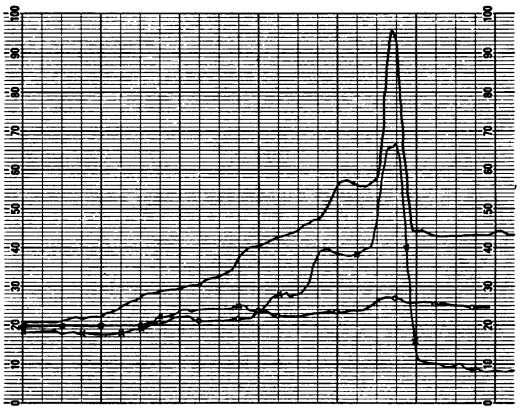

FIG. 3. *Composite graph showing the concentration of areas and number of zones for VSB supernatant* (○), *GSB*(—), *and Escherichia coli* (×) *guinea pig supernatant fluids against antiserum to GSB supernatant in Oudin columns. Recording made after 5 days at 25 C. Final dilution of serum was 1:2.*

number of zones of precipitation due to nonspecific guinea pig protein in the in vivo supernatant. Antisera to VSB supernatant produced no reaction with the three supernatant fluids and, therefore, was not included in the table. Figure 3 is a composite graph of the tracings of a photron-reflectometer measuring the number of zones and concentration of areas of the same external reactants vs. anti-GSB supernatant. From Table 5 and Fig. 3, it is evident that a minimum of two precipitation bands are produced by staphylococcal soluble products in the GSB supernatant. Presumably, these soluble products are not present in the VSB supernatant. It is also evident that three nonspecific zones are produced by guinea pig soluble antigens carried in the in vivo supernatant fluids.

Tracings of the reaction of VSB and GSB supernatant fluids and controls vs. anti-GSB supernatant by the Ouchterlony technique are depicted in Fig. 4. A minimum of two and possibly three bands peculiar to staphylococcal products is evident around the fourth antigen well. The antigens used in the fourth and third well represent fresh isolates taken immediately before the Ouchterlony plate was set up, whereas the antigens in wells one and two were isolated a few months prior to the test. It is possible that their staphylococcal elements were destroyed in storage. As in the Oudin tubes, the Ouchterlony plates showed that staphylococcal products were

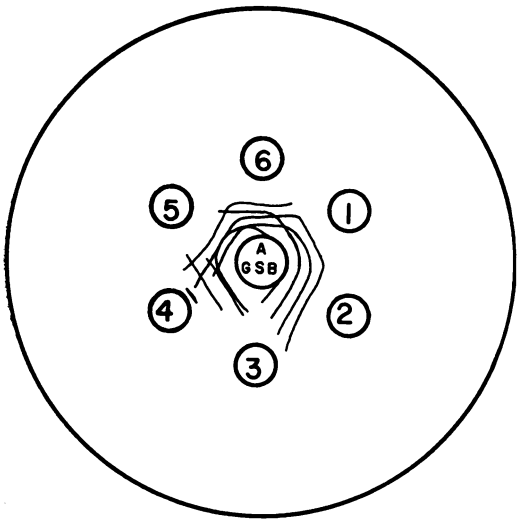

FIG. 4. *Reaction in Ouchterlony plate of anti-GSB supernatant with various antigens. The serum (anti-GSB supernatant) was in the center wall. The following antigens were in the numbered wells:* (1) *GSB supernatant, isolate a;* (2) *GSB filtered suspension;* (3) *VSB supernatant;* (4) *GSB supernatant, isolate b;* (5) *guinea pig complement; and* (6) *Escherichia coli guinea pig supernatant.*

in the GSB but not in the VSB supernatant fluids. Figure 4 also portrays well the reaction of identity due to guinea pig protein in the GSB supernatant, complement, and *E. coli* guinea pig supernatant fluids.

Virulence studies. To compare the lethal properties of the VSB and GSB strains, groups of five mice were injected intraperitoneally with the bacterial cells or their supernatant fluids. None of the mice could be killed with the supernatant of VSB, whereas in 2 days five of ten mice injected with a 1:5 dilution of the GSB supernatant were killed. When living cells of the two strains were injected intraperitoneally into mice, 2×10^9 VSB cells and only 9×10^7 GSB cells were needed to insure 100% mortality.

In the course of this work and another problem under investigation in this laboratory, a total of 39 rabbits were injected intravenously with the living VSB and GSB strains. Of 20 rabbits receiving the GSB strain, 19 developed an acute osteomyelitis within 10 days. Each rabbit had received, at most, 3.4×10^8 cells by this time. Debilitation, incontinence, and death would have followed if chloramphenicol had not been administered. Autopsy of dead animals showed multiple abscesses in the liver, spleen, and cardiac muscle but not in the kidneys. Of the 19 rabbits receiving the VSB cells, 9 developed an osteomyelitis, but not before 3 to 4 weeks; by that time, these animals had received two to three times more cells than did the rabbits receiving the GSB cells.

To contrast the invasive and dermonecrotic abilities of the VSB and GSB strains, a few rabbits were injected intradermally with 0.1 ml of the cells or their supernatant fluids. The site of injection with the VSB supernatant fluid exhibited only a slight erythema by 24 hr; this lesion did not progress, and all traces of it had disappeared by 4 days. However, the injection of GSB supernatant produced an intense spreading necrosis of the skin which, within 48 hr, had progressed to a brown punched-out ulcer (2 cm in diameter), a reaction typical of the dermonecrotizing toxin. This effect was completely neutralized in rabbits by known antidermonecrotic serum and also by human γ-globulin when passively administered. Within 24 hr, the intradermal injection of 2×10^6 live GSB cells produced a blanched area (4 cm in diameter) which progressed by 4 days to a raised, indurated lesion with a punched-out center. In time, the punched-out area became necrotic and resembled the effect of the necrotizing toxin. When 5.2×10^7 VSB cells were injected intradermally, only a very slight area of erythema developed in 24 hr; within 4 days, this intensified and was followed by the appearance of yellow pus and softening in the center of the lesion, the pattern of a typical furuncle.

DISCUSSION

Gellenbeck (1962) reported that staphylococci recovered from an infected animal differed significantly in respiratory rate when compared with the same culture grown in vitro. The present work shows that the two strains, VSB (in vitro) and GSB (in vivo), differed significantly in characteristics other than respiratory rate. An extensive series of tests applied to VSB and GSB were equivocal. These procedures included the usual characteristics of taxonomic significance and the usual tests employed in clinical work to determine potential pathogenicity. The in vivo cells differed from the in vitro cells in at least eight particular ways. The in vivo strain quanti-

tatively produced more deoxyribonuclease, α-hemolysin, leucocidin, and hyaluronidase than did the in vitro strain. The GSB cells differed from VSB cells in agglutinability and in virulence potential. Strain GSB failed to grow on tellurite-glycine agar; strain VSB grew well. Human γ-globulin markedly inhibited growth of GSB organisms, whereas VSB cells were inhibited to a lesser degree.

The dramatic difference in amount of α-hemolysin, leucocidin, hyaluronidase, and deoxyribonuclease in the GSB supernatant fluid over that in the VSB supernatant is significant. To our knowledge, no single artificial medium exhibits the same efficiency. Studies are in progress to determine how many and how much of the various staphylococcal products can be found in the VSB supernatant fluid after manifold concentration of this fluid. Whether the presence of these cellular products has any causal relation to, or is merely concurrent with, the property of virulence is still uncertain. However, Anderson (1956) found a direct relationship between the number of flocculation lines from soluble products of staphylococci and the severity of the staphylococcal lesion.

Although both VSB and GSB strains were bacteriophage-type 80/81, tube-agglutination tests indicated a marked difference in agglutinability of the two strains when tested in the same antiserum. Antisera prepared against Zephiran-killed GSB organisms agglutinated GSB cells well (titer, 1:320) but agglutinated VSB cells poorly (titer, 1:10). Antisera to living GSB antigen yielded strong, granular agglutination of the VSB antigen in dilutions as high as 1:5,120, but the same antisera gave "thready" agglutination of GSB cells in dilutions as high as 1:320. "Thread" agglutination in the enteric organisms is a manifestation of O-inagglutinability (Stuart, Feinberg, and Feinberg, 1948) and is frequently associated with the virulence of the organism. It is possible that strain GSB could represent a serotype distinct from strain VSB, but it is just as possible that, along the lines of the finding of Brodie, Guthrie, and Sommerville (1958), strain VSB may represent a less pathogenic (more agglutinable) member of the same serotype as strain GSB. Recently, Pereira (1961) reported that most newly isolated strains of *S. aureus* possessed one of the antigens 13, 17, or 18; on continuous subculture in vitro, the variation of antigen 13 → 3 and 17 → 1 was very often observed. The VSB strain was never subcultured in vitro as often as Pereira found necessary to effect this antigenic variation. Since GSB antiserum was directed against the in vivo strain, the agglutination results may be detecting the same progressive antigenic fluctuation. Pereira found no change in phage type in a strain before and after antigenic variation.

The virulence studies do not allow the conclusion that the VSB strain is avirulent and the GSB strain virulent, but they do indicate that the degree and possibly the type of virulence of strain GSB is quite distinct from that of strain VSB.

The inability of the VSB supernatant fluid to induce a pathological situation by the intraperitoneal or intradermal routes may simply reflect the fact that enough soluble products were not produced by this method. Death of mice following intraperitoneal injection of the two strains may have been brought about by an overwhelming administration of foreign protein, but more likely a definite number of cells (always fewer of the GSB strain) are needed to guarantee enough preformed α-hemolysin. A different type of virulence seems to be in evidence with the intravenous injection of live cells into rabbits. The fact that we were able to induce death in rabbits by this route faster and with fewer cells when the GSB strain was used would seem to us to be convincing evidence of the greater virulence or the distinctness in type of virulence of the GSB strain. Smith and Dubos (1956) came to a somewhat similar conclusion. In working with infections in mice, they found the difference in virulence between coagulase-positive and coagulase-negative strains to be quantitative rather than qualitative; in fact, they found that various strains could be arranged in a continuous spectrum with many intermediate degrees of virulence.

Hoeprich, Croft, and West (1960) and Burns and Holtman (1960) recommended the use of tellurite-glycine agar and a minimal agar, respectively, as a means of isolating potentially pathogenic staphylococci and of excluding nonpathogenic strains. Our work confirms this only if the nonpathogenic strain belongs to the *S. epidermidis* group. On the other hand, we find that these two media selectively favor the growth of the less virulent strain (VSB) over the more

virulent strain (GSB). This disparity in our findings and theirs is cause for serious concern, in that the use of certain media may actually prevent one from isolating staphylococci which resemble strain GSB. The fact might imply a new approach in the isolation of the more virulent staphylococcal colonies from pathological situations. One such approach could be a quasi replica plating on enriched, as well as tellurite-glycine and minimal, agar. Strains, which would not grow on the latter media, could be picked from the enriched medium. The application of the replica-plating technique of Runnels and Wilson (1960) would be a step in the right direction if we could avoid the incubation in beef-infusion broth before plating on the indicator or selective media. Even one passage in artificial medium seems to accelerate the emergence of a less virulent population in a strain grown originally in vivo.

The results described here indicate that human γ-globulin exhibits a greater bactericidal effect in agar against GSB than it does against VSB and *S. epidermidis* strains. To the best of our knowledge, this phenomenon has not been reported to date. Staphylococci could not be cultivated from the area of inhibition. We could not demonstrate this bactericidal effect with rabbit sera prepared against GSB living cells, GSB supernatant fluid, or with commercial horse anti-α-toxin. The effect does not seem to be due to the human-serum antistaphylococcal factor, described by Yotis and Ekstedt (1959), because the inhibitory effect was greater against the coagulase-positive than against the coagulase-negative strains. It seems possible that the differential inhibitory effect of human γ-globulin may be correlated with the kind and the frequency of staphylococcal infection. For example, the kind of staphylococcal infection may manifest itself (in order of increasing severity and decreasing frequency) as a single self-limiting furuncle, multiple and chronic furunculosis, osteomyelitis, generalized septicemia with or without organ involvement, and as a systemic, lethal infection which is rapidly fulminating. The present work shows that, at least in animals, the strain with the greater (systemic) virulence potential was more susceptible to the bactericidal action of γ-globulin than was the strain with the lesser virulence.

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