JOURNAL OF BACTERIOLOGY, Sept., 1966
Vol. 92, No. 3

Significance of Chromogenic Variants in Studies of Virulence Factors of *Staphylococcus aureus*

JOSEPH T. PARISI[1]

Department of Biological Sciences, Duquesne University, Pittsburgh, Pennsylvania

Received for publication 21 April 1966

ABSTRACT

PARISI, JOSEPH T. (Duquesne University, Pittsburgh, Pa.). Significance of chromogenic variants in studies of virulence factors of *Staphylococcus aureus*. J. Bacteriol. 92:589–591. 1966.—Large numbers of chromogenic variants were isolated from cultures of a parent strain of *Staphylococcus aureus* growing in Brain Heart Infusion (Difco). The parent strain and four selected chromogenic variants were tested for either quantitative or qualitative differences in the production of extracellular substances associated with virulence. Quantitative differences were found in the ability of these strains to produce coagulase and hyaluronidase, whereas qualitative differences were found in the production of plate hemolysins, bound coagulase, opacity in an egg yolk medium, and a proteinase. In view of the rate and extent of the occurrence of these chromogenic variants, their presence in an inoculum could lead to inaccurate results in in vitro studies of staphylococcal virulence.

Numerous investigators (4, 5, 7) have observed the occurrence of spontaneous variants of *Staphylococcus aureus* with altered ability to produce extracellular substances associated with virulence. However, to our knowledge, there are no reports showing the rate and extent of such changes in common laboratory media. Pigmentation was chosen as the parameter of population changes in *S. aureus*. This study presents quantitative data of these population changes. It also was designed to determine whether chromogenic variants differ in the quantitative or qualitative production of virulence factors.

MATERIALS AND METHODS

Bacterial strains. The parent strain, *S. aureus* 6, was originally isolated from a human infection. Its phage type was 80/81, and it produced a golden-yellow pigment. Spontaneous chromogenic variants were isolated from Brain Heart Infusion (BHI; Difco) Agar or broth cultures of the parent strain, which were diluted in distilled water and plated on Trypticase Soy Agar (BBL) containing 20% (v/v) nonfat milk (milk-agar). Plates were incubated at 37 C for 24 hr and then at 25 C for 48 hr. Any colony with a pigment that differed obviously in color or intensity from the parent strain and which phage-typed 80/81 was considered a variant. The parent strain and four selected variants were used in the studies of virulence factors. Strain 6A produced a light-yellow pigment; 6B, a white pigment; 6C, a golden-yellow pigment more intense than the parent strain; and 6D, a yellow-green pigment.

Chromogenic variants in BHI broth. A single isolated colony of the parent strain on milk-agar was inoculated onto a BHI agar slant and was incubated for 18 to 24 hr at 37 C. Growth from the slant was suspended in distilled water and washed and centrifuged three times; the suspension was then standardized with a Klett-Summerson colorimeter. Approximately 10^4 cells per milliliter of medium were inoculated into triplicate tubes of BHI broth and were incubated at 37 C. After 4, 8, and 12 days, appropriate dilutions in distilled water were made from each tube and were streaked on milk-agar plates with glass spreaders. After incubation at 37 C for 24 hr, and then at 25 C for 48 hr, the number of parent and chromogenic variant colonies was determined. Representative chromogenic variant colonies were subsequently phage-typed.

Production of virulence factors. The method of Elek and Levy (2), with some modification, was used for the production and quantitative determination of α hemolysin. BHI broth containing 0.3% agar was the growth medium, incubation was at 37 C for 48 hr in an atmosphere of 80% CO_2, and the supernatant fluids were sterilized by filtration through an O2 Selas filter. Plate hemolysins were determined by streaking each strain radially onto Heart Infusion Agar (BBL) containing rabbit, sheep, or human erythrocytes, and then incubating for 24 and 48 hr at 37 C in an atmosphere of 20% CO_2.

The production and quantitative determination of coagulase was done according to the method of

[1] Present address: Department of Microbiology, School of Medicine, University of Missouri, Columbia.

Tager and Hales (8), except that the supernatant fluids were sterilized by filtration through a 0.45-μ membrane filter (Millipore), and sterile citrated rabbit plasma was used. Coagulase production was also determined by the tube method (1), and bound coagulase was determined by the slide method (1).

The production of hyaluronidase was according to the method of Rogers (6), except that veal and 1% peptone (Difco) was the growth medium, and the supernatant fluids were sterilized by filtration through a 0.45-μ membrane filter (Millipore). The turbidimetric method (9) was used for the estimation of hyaluronidase. Hyaluronic acid, grade I, and type I hyaluronidase (Sigma Chemical Co., St. Louis, Mo.) were used in the standard assay.

RESULTS

Growth of the parent strain in BHI broth for various periods of time resulted in the emergence of spontaneous chromogenic variants. As shown in Table 1, chromogenic variants were the predominant organisms in two of the three samples after 8 days and were present in overwhelming number in all three samples after 12 days.

The production of virulence factors by the parent and four selected variant strains is shown in Table 2. Although there were no differences in serial twofold dilutions of cell-free filtrates of α hemolysin, on agar media, strain 6A hemolyzed rabbit erythrocytes more slowly and failed to hemolyze human erythrocytes. All strains produced α hemolysin on agar plates containing sheep erythrocytes.

As shown in Table 2, not only were differences observed in the amount of coagulase produced by the parent and variants, but cell-free filtrates of strain 6B did not have detectable amounts of coagulase. Although cultures of 6B clot plasma within 3 hr, the coagulum liquefies within 24 hr. Apparently, this organism produces a proteinase that destroys coagulase. The parent and variant strains 6A, 6C, and 6D produced bound coagulase as determined by the slide method, but 6B did not. Since 6A, whose pigment is light-yellow, produced the weakest clumping reaction for bound coagulase, and 6B, whose pigment is white, failed to produce bound coagulase, it appeared that the intensity of this clumping reaction may be associated with the intensity of the color of the pigment. To determine this, 34 phage-distinct strains of *S. aureus*, positive for coagulase production by the tube method, were tested for bound coagulase production. Twelve of 13 golden-yellow pigmented strains, 6 of 9 light-yellow pigmented strains, and 4 of 12 white pigmented strains produced bound coagulase. These results indicate a high correlation between the production of bound coagulase and the intensity of the color of the pigment.

Table 2 also shows differences in the amount of hyaluronidase produced by the parent and variants. Differences were observed also with different stock cultures (not shown) of the parent strain. On the other hand, no qualitative differences were observed in the production of fibrinolysin, deoxyribonuclease, gelatinase, phosphatase, or growth on mercuric chloride-agar, but differences were observed with strains 6B and 6C in the rate and type of opacity produced in an egg-yolk medium (3), and only 6B produced a proteinase on milk-agar. Interestingly, the results of virulence tests showed that all strains were equally virulent for mice when injected intraperitoneally with approximately 6×10^8 washed cells and 0.5 ml of mucin.

TABLE 1. *Occurrence of chromogenic variants of Staphylococcus aureus in Brain Heart Infusion broth*

Tube no.	Ratio of variants to parent detected after incubation for		
	4 days	8 days	12 days
1	ND[a]	0.01	2.8
2	0.02	2.3	4.2
3	0.01	1.3	25.0

[a] None detected.

TABLE 2. *Production of hemolysins, coagulase, and hyaluronidase by parent and variant strains of Staphylococcus aureus*

Strain	Hemolysin on agar plates containing erythrocytes of		Reciprocals of titers of		Hyaluronidase (turbidity-reducing units per ml of medium)
	Rabbit	Human	α-Hemolysin	Coagulase	
6	α	Δ	400	4	2.0
6A	α[a]	None	400	16	0.5
6B	α	Δ	800	ND[b]	3.6
6C	α	Δ	400	2	1.5
6D	α	Δ	400	2	1.8

[a] Produced after 48 hr.
[b] Not detectable.

DISCUSSION

The occurrence of large numbers of spontaneous chromogenic variants in BHI broth indicates that these variants appeared to have a selective advantage in this medium. The mutant frequency of chromogenic variants in a 24-hr BHI slant culture of the parent strain was

observed in our laboratory to be 19 per 5×10^4 cells. The inoculum for this slant was a single isolated colony on milk-agar. The mutant frequency observed indicates the proportion of chromogenic variants present in a culture of the parent strain. Because of this relatively high mutant frequency, and the rapid and extensive population changes which occurred after growth in BHI broth, the presence of these spontaneous variants in cultures of *S. aureus* should be brought to the attention of investigators working with this organism.

The studies on the production of virulence factors confirm the reports of others of the occurrence of spontaneous variants of *S. aureus*, and, more specifically, of chromogenic variants, often with a concurrent altered ability to produce some extracellular substances associated with virulence. The differences observed with some variants in the production of Δ hemolysin, coagulase, hyaluronidase, and egg-yolk opacity show the need of working with relatively homogenous cultures of this organism. In view of the rate and the extent of the occurrence of these variants, their presence in an inoculum could lead to inaccurate results in studies of staphylococcal virulence in vitro.

ACKNOWLEDGMENTS

The technical assistance of Elaine A. Parisi and Howard T. Berkins is gratefully acknowledged.

This investigation was supported by grant F-13 from the Health Research and Services Foundation of Pittsburgh, Pa.

LITERATURE CITED

1. BAILEY, W. B., AND E. J. SCOTT. 1966. Diagnostic microbiology, p. 106. C. V. Mosby Co., St. Louis.
2. ELEK, S. D., AND E. LEVY. 1954. The nature of discrepancies between hemolysins in culture filtrates and plate hemolysin patterns of staphylococci. J. Pathol. Bacteriol. **68**:31–40.
3. GILLESPIE, W. A., AND V. G. ALDER. 1952. Production of opacity in egg yolk media by coagulase-positive staphylococci. J. Pathol. Bacteriol. **64**:187–200.
4. HAQUE, R., AND J. N. BALDWIN. 1964. Types of hemolysins produced by *Staphylococcus aureus*, as determined by the replica plating technique. J. Bacteriol. **88**:1442–1447.
5. KOENIG, M. G., AND M. A. MELLY. 1965. The importance of surface antigens in staphylococcal virulence. Ann. N.Y. Acad. Sci. **128**:231–250.
6. ROGERS, H. J. 1953. Variant populations within a hyaluronidase-producing culture of *Staphylococcus aureus*. J. Pathol. Bacteriol. **66**:545–551.
7. ROGERS, H. J. 1956. The formation of extracellular enzymes by staphylococci. Ann. N.Y. Acad. Sci. **65**:132–138.
8. TAGER, M., AND H. B. HALES. 1947. Quantitative coagulase and toxin production by staphylococci in relation to the clinical source of the organisms. Yale J. Biol. Med. **20**:41–49.
9. TOLKSDORF, S. 1954. The *in vitro* determination of hyaluronidase. Methods Biochem. Anal. **1**:425–457.