INFECTION AND IMMUNITY, Nov. 1970, p. 523-527

Vol. 2, No. 5
Printed in U.S.A.

Comparison of Compact and Diffuse Variants of Strains of *Staphylococcus aureus*

KOSAKU YOSHIDA AND YOSHIO TAKEUCHI
Department of Microbiology and Immunology, Nippon Medical School, Tokyo, Japan

Received for publication 3 June 1970

Several biological characteristics of six diffuse-type variants and six compact-type variants of *Staphylococcus aureus* were compared on the basis of morphology, phage type, certain cellular products, growth rate, and mouse virulence. All compact variants gave a positive test for clumping-factor reaction, coagulase, deoxyribonuclease, mannitol fermentation, hemolysis, and pigmentation. Four of the six compact variants were phage typable and lacked capsules. None of the diffuse variants was phage-typable or possessed the clumping-factor reaction. Only one diffuse variant had hemolytic activity, and all of the diffuse strains were encapsulated. No differences between the compact and diffuse strains were noted with respect to mannitol fermentation or deoxyribonuclease activity. Coagulase and acid phosphatase activities of the culture supernatant fluid were diminished in most of the diffuse strains. Less β-carotene content was found in cells of diffuse variants. Virulence in mice was found to correlate with the capsule size, as the mortality rate was greatest for diffuse variants having the largest capsules. Growth rates of variants were generally not significantly different.

Since the report of Hunt and Moses (4), the Smith strain has become the focus of many studies on *Staphylococcus aureus*. This strain is considered the representative type of encapsulated strains of *S. aureus* (8). Additional encapsulated strains have been reported by Wiley (11) and Iwata and Eda (5). In a previous paper, the authors (15) described the procedure for isolation of diffuse variants by a serum-soft agar technique. The isolation of four diffuse and four compact variants from a single colony was achieved.

The purpose of this report is to characterize both compact and diffuse variants isolated from six different encapsulated strains of *S. aureus*.

MATERIALS AND METHODS

Strains of Staphylococcus aureus. Of the six encapsulated strains used in these experiments, two came from R. D. Ekstedt (Dept. of Microbiology, Northwestern Univ., Chicago, Ill.). The first of these two was originally supplied by M. G. Koenig (Vanderbilt Univ., Nashville, Tenn.) and the second originated at Ohio State Univ., Columbus, Ohio. These strains are designated Smith-Koenig and Smith-OSU. Strain 334 was described elsewhere (15). Strains YT-6, YT-35, and YT-36 were isolated from human clinical specimens.

Media. Cultures were grown in Brain Heart Infusion (BHI; Difco), Trypticase Soy Agar (Difco), or modified Staphylococcus 110 medium as previously described (15).

Isolation procedure for diffuse variants. The isolation procedure for diffuse variants was reported in an earlier paper (13). These were true diffuse, encapsulated strains.

Examination for metabolic products. All strains were cultured in modified Staphylococcus 110 broth for 5 days at 37 C. The cells were removed by centrifugation, and the supernatant fluids were used for the examinations. Coagulase activity was measured by mixing 0.5 ml of rabbit plasma, diluted 1:5 with M/15 phosphate-buffered saline containing 1:5,000 Merthiolate (Takeda Pharmaceutical Co., Ltd., Osaka, Japan), with 0.5 ml of culture supernatant. The samples were then placed in a water bath at 37 C, and the results were recorded after 2, 4, and 8 hr of incubation. The method of Kamijo and Inoue (7) was employed for the determination of deoxyribonuclease activity. Acid phosphatase activity was measured by the procedure of Barnes and Morris (1); one unit is defined as that amount of enzyme necessary to produce 1 μmole of *p*-nitrophenol in 1 min. The clumping-factor reaction was measured with overnight culture of the strains on BHI Agar plate by a standard method which used normal rabbit serum diluted 1:10 with M/15 phosphate-buffered saline. The types of hemolysin were identified by the method of Christie et al. (3) by using sheep blood-agar. Milk-salt-agar plates incubated at 37 C and read at 24 and 48 hr were used for the determination of pigmentation. For determination of intracellular β-carotene, cultures were grown on modified Staphylococcus 110 agar for 2 days at 37 C and were subsequently maintained for 5 more days at 25 C; pigment

was then eluted with acetone and the β-carotene was estimated photometrically (14).

Phage typing. Twenty-five staphylococcal phages were provided by M. R. Smith, U.S. Army, Kanagawa-ken, Japan. Cultures were phage typed by the method of Blair and Carr (2).

Growth curves. All strains were cultured overnight in modified Staphylococcus 110 broth. A 0.1-ml amount of the cultures was transferred into L-form tubes containing 15 ml of the same medium and was then incubated in a shaking-water bath at 37 C. Absorbancy at 420 nm was observed every hour for 12 hr with a spectrophotometer (model II, Tokyo Riko, Co., Ltd., Tokyo, Japan).

Cell volume index. Cells were harvested after 18 hr of growth at 37 C in plates of BHI Agar. After one washing with saline, they were suspended in the same solution. Portions (3 ml) were transferred to a Hopkins tube, and packed cell volumes were noted after centrifugation at 3,500 × *g* for 20 min in a Tominaga centrifuge (model SV 60). The number of colony-forming units (CFU) was estimated from plates made from the packed cells which had been shaken with glass beads in 50-ml sterile flasks. Cell volume indices were calculated by using the following formula: [packed cell volume (ml)/CFU] × 10^{10}.

Capsule staining. The presence of capsules was observed with the light microscope after staining prepared smears with India ink.

Pathogenicity testing. Cell suspensions for injection into mice were prepared from cultures grown on modified Staphylococcus 110 agar. The organisms were gently washed three times with modified Staphylococcus 110 broth and suspended in the same medium to give an optical density $(OD)_{420\ nm}$ of 0.5. These cell suspensions contained 2.2 × 10^8 to 5.1 × 10^8 CFU/ml. Tenfold serial dilutions of the cell suspensions were prepared, and 0.5 ml of each dilution was injected intraperitoneally (ip) into each of ten 20-g white mice (Shiihaski Farm, Tokyo, Japan.) The death rate was determined during a 2-week period.

RESULTS

Morphological relationship with the clumping-factor reaction. All compact strains exhibited positive clumping-factor reaction and were not encapsulated (Table 1). None of the diffuse variants exhibited positive clumping-factor reaction, but capsules were evident. Therefore, it appears that diffuse colony morphology of cells possessing capsules is associated with the absence of a clumping-factor reaction. Pigments produced on milk-salt-agar by the compact variants were either orange or yellow, whereas those produced by diffuse variants varied from white to yellow. Three of the strains, Smith-Koenig, Smith-OSU, and 334, produced yellow pigment in both the compact and diffuse variants. However, diffuse variants of the clinical isolates, YT-6, YT-35, and YT-36, all produced lighter colored colonies than those produced by their corresponding compact variants.

TABLE 1. *Relationship between pigmentation and the clumping-factor reaction of compact and diffuse variants of Staphylococcus aureus*

Strain	Growth type[a]	Clumping-factor reaction	Pigment color	β-carotene
				μg/mg
Smith-Koenig	Compact	+	Yellow	0.057
	Diffuse	−	Yellow	0.083
Smith-OSU	Compact	+	Yellow	0.024
	Diffuse	−	Yellow	0.015
334	Compact	+	Yellow	0.048
	Diffuse	−	Yellow	0.018
YT-6	Compact	+	Yellow	0.043
	Diffuse	−	White	0.053
YT-35	Compact	+	Orange	0.187
	Diffuse	−	Cream	0.008
YT-36	Compact	+	Orange	0.002
	Diffuse	−	Cream	0

[a] Description of growth development in Brain Heart Infusion serum-soft agar.

The concentration of β-carotene was determined for each variant because it was considered the source of pigmentation. A slightly higher content of β-carotene was found in the diffuse variants of Smith-Koenig and YT-6 than in those of the corresponding compact colonies. Compared with the compact variants, all but one of the remaining diffuse variants had significantly lower levels. There appeared to be a lack of correlation between β-carotene content and pigment production.

Phage type. Four of the six compact variants were phage typable (Table 2). However, none of the diffuse variants could be typed. The dissimilarity of the Smith-Koenig and Smith-OSU strains was revealed by their phage typing. The former strain was type 29, whereas the latter strain was not typable. Both of these strains were also distinguishable from the original Smith strain type 44A/42E (3, 9). In initial studies, we found that the diffuse variants of Smith-Koenig, YT-35, and YT-36 reverted to the phage pattern characteristic of their compact variants after several subcultures on BHI Agar. Phage patterns of the other diffuse variants remained stable under similar growth conditions.

Presence of other staphylococcal products. Coagulase, deoxyribonuclease, and acid phosphatase activities of the various strains studied are given in Table 2. All of the compact variants

TABLE 2. *Relative enzyme activity of compact and diffuse variants of Staphylococcus aureus*

Strain	Growth type[a]	Phage type	Coagulase (titer)	Deoxyribonuclease (absorbance at 260 nm)	Acid phosphatase (units[b])	Toxin type[c]
Smith-Koenig	Compact	29	1:128	0.178	0.011	β, δ
	Diffuse	NT[d]	1:64	0.148	0.008	
Smith-OSU	Compact	NT	1:4	0.272	0.011	β, δ
	Diffuse	NT	None	0.171	0.011	
334	Compact	187	1:128	0.211	0.011	α
	Diffuse	NT	1:256	0.149	0.009	α
YT-6	Compact	NT	1:64	0.248	0.018	α
	Diffuse	NT	None	0.205	0.011	
YT-35	Compact	71	1:16	0.352	0.009	β, δ
	Diffuse	NT	1:32	0.263	0.009	
YT-36	Compact	71/55	1:128	0.170	0.009	α
	Diffuse	NT	1:1	0.190	0.005	

[a] Description of growth development in Brain Heart Infusion serum-soft agar.
[b] Expressed as micromoles of *p*-nitrophenol per minute per milliliter of culture supernatant.
[c] Determined by the CAMP test of Christie et al. (3).
[d] Not typable.

TABLE 3. *Relationship of cell volume index and mortality rate of mice with diffuse variants of Staphylococcus aureus*

No. of organisms injected[a]	Diffuse variant[b]					
	Smith-Koenig	Smith-OSU	334	YT6	YT35	YT36
10^1	4/10	3/10	2/10	NT[c]	NT	NT
10^2	7/10	8/10	4/10	4/10	NT	NT
10^3	10/10	9/10	6/10	10/10	2/10	1/10
10^4	10/10	9/10	9/10	10/10	4/10	4/10
10^5	10/10	10/10	10/10	10/10	6/10	7/10
10^6	NT	NT	NT	NT	8/10	8/10
Cell volume index[d]	10.52	10.82	7.08	11.19	6.12	6.40

[a] Mice were injected ip with 0.5 ml of a cell suspension containing 5% mucin.
[b] Number dead/number injected.
[c] Not tested.
[d] Calculated as described in Materials and Methods.

had coagulase, deoxyribonuclease, and acid phosphatase activity. The diffuse variants, Smith-OSU and YT-6, were negative for coagulase activity, and the deoxyribonuclease activity was significantly lower than for their corresponding compact variant. In most cases, the coagulase and deoxyribonuclease activities of the diffuse variants were less than those of the compact variants. Exceptions, however, were noted with the 334 and YT-36 strains. The acid phosphatase activity of diffuse variants, with the exception of strain Smith-OSU, was significantly lower than that of the compact variants.

All of the variants studied, compact or diffuse, were positive for mannitol fermentation (*not tabulated*).

Compact variants produced either α or β and δ toxins. Only one of the diffuse variants, 334, produced toxin in this experimental system. The latter variant produced α toxin as did the corresponding compact strain.

Mortality rate in mice and cell volume index. A striking difference in the susceptibility of mice to certain diffuse variants was noted (Table 3). The YT-35 and YT-36 variants were far less lethal than those of Smith-Koenig, Smith-OSU, and YT-6.

A cell volume index for each diffuse variant

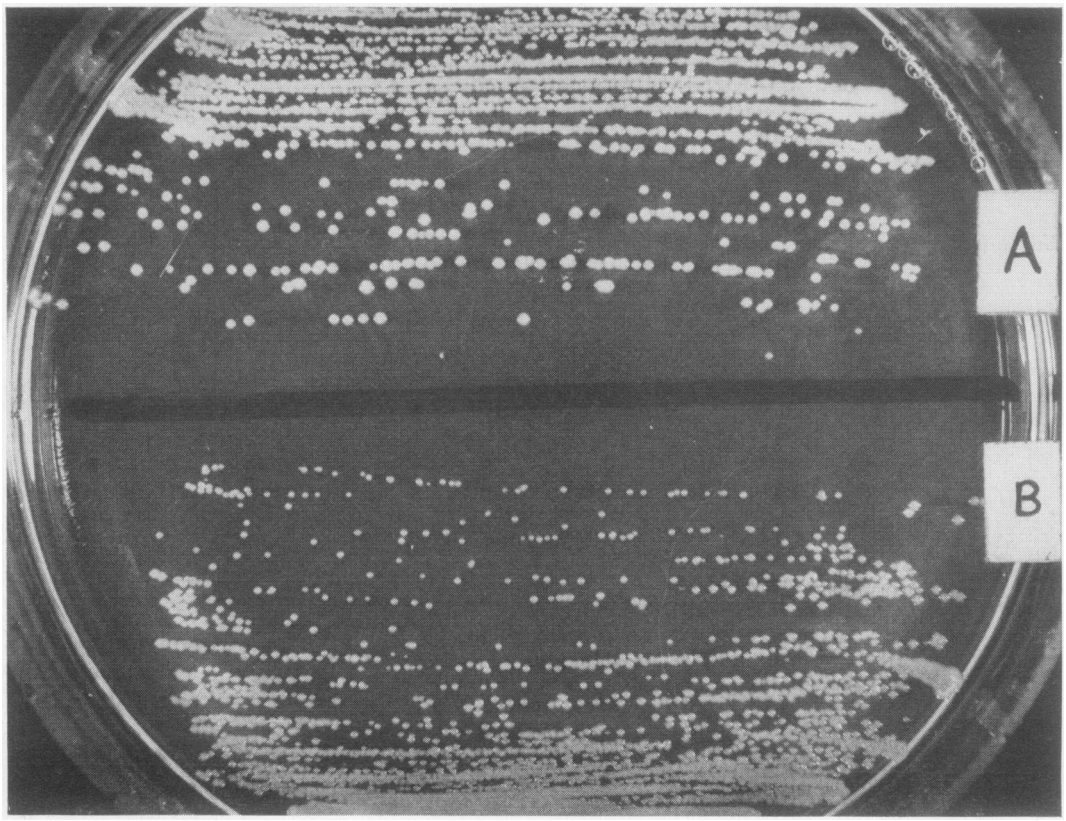

FIG. 1. *Comparison of colony size of diffuse variant 334 (A) and corresponding compact variant (B)*

was obtained. The results showed that the variants with the largest capsules, YT-6, Smith-OSU, and Smith-Koenig, were the most virulent. Diffuse variant 334, having a middle-sized capsule, was slightly less virulent. The YT-35 and YT-36 diffuse variants were clearly the least virulent and also possessed the smallest capsules. Incidently, the cell volume indices of the compact organisms ranged from 4.72 to 5.46. The diameters of the largest and smallest capsules possessed by the diffuse variants YT-6 and YT-35 were 2.02 and 1.29 times larger, respectively, than their compact counterparts. Thus, the degree of virulence of the diffuse variants corresponded well with the capsule size (Table 3).

A remarkable increase in the size of the colonies of diffuse variant 334 over compact variant was observed by subculture on modified Staphylococcus 110 Agar. Diameters of the diffuse and compact colonies were 2.08 to 2.48 and 0.94 to 1.08 mm, respectively, after overnight culture on the plate. Rough colonies were not apparent as in pneumococci. This phenomenon was also noted in strain YT-6 but not in strains YT-35 and YT-36.

Growth rates of variants. Growth curves of all six of the compact variants and their diffuse variants were compared (*not illustrated*). Generally, the growth curves for the diffuse variants closely resembled those of their related compact variants. However, differences were noted in the YT-35 and YT-36 strains whose diffuse variants gave longer generation times.

DISCUSSION

For several decades, toxins and extracellular enzymes produced by *S. aureus* were considered to be directly related to the establishment and progress of staphylococcal infection. On the other hand, Koenig (7) has emphasized the role of the capsule of the Smith strain in staphylococcal infection. Iwata and Eda (4) demonstrated that special strains of the diffuse type were coagulase-negative and deoxyribonuclease-positive, but still virulent for mice. In our present study, diffuse variants which were isolated from single colonies derived from compact strains exhibited lower coagulase, deoxyribonuclease, acid phosphatase, and hemolysin activities than

the original compact strains. The β-carotene content of the diffuse strain was also reduced. Furthermore, diffuse variants were encapsulated, lacked the clumping-factor reaction, and were not phage typable. Two of the diffuse variants, Smith-OSU and YT-6, did not exhibit positive clumping-factor reaction, coagulase, or hemolysin, but did produce high mortality rates in mice. Our results suggest that mouse virulence is not directly related to the production of such cellular products as clumping factor, deoxyribonuclease, acid phosphatase, hemolysin, or pigment. Our investigation, however, does indicate that mouse virulence is directly related to the capsule and its size. These results are similar to those reported by Wiley (11), except that he noted increased coagulase activity with his encapsulated strain.

Genetic mutations of *S. aureus* have resulted in the production of variants avirulent for mice. Yoshida (12) developed, in the presence of progressive concentrations of tetracycline, a mutant which was resistant to the antibiotic and lacked the clumping-factor reaction, coagulase, and hemolysin. Jungerman and Lipnicki (6) produced, by ultraviolet irradiation, an avirulent mutant which was negative for both clumping-factor reaction and coagulase. These mutants are not encapsulated and, therefore, differ from the diffuse-type strains of encapsulated organisms included in our investigation.

The more rapid growth rate of two of the compact strains over their counterpart diffuse strains could be a disadvantage in the isolation of diffuse mutants from laboratory cultures and natural specimens. Further, the isolation of diffuse variants is complicated by the transition from the diffuse state to a compact state through a "pseudocompact type" (15). Consequently, the capsule of certain strains is labile.

In the present study, three diffuse variants (YT-6, YT-35, and YT-36) were obtained from 40 fresh isolates of *S. aureus*. By using the isolation methods of this investigation, we have considerably increased the percentage of diffuse mutants isolated over that reported by Rogers (9), who was able to isolate only 7 diffuse strains from 1,800 strains of *S. aureus*. It is possible that with more efficient methods the percentage of diffuse strains isolated would further increase. Such increased isolation would perhaps more closely reflect the true population of diffuse strains in clinical infections.

ACKNOWLEDGMENTS

The authors are grateful to Y. Kimura, Department of Microbiology and Immunology, Nippon Medical School, Tokyo, Japan, for his advise and encouragement and to C. L. San Clemente, Department of Microbiology and Public Health, Michigan State University, for assistance in preparing the manuscript.

Strains of *S. aureus* and typing bacteriophages employed in this study were kindly supplied by T. Kitajima, Kitajima Clinic, Tokyo, Japan; R. D. Ekstedt, Department of Microbiology, Northwestern University; and M. R. Smith, U. S. Army, Japan.

LITERATURE CITED

1. Barnes, E. H., and J. F. Morris. 1957. A quantitative study of the phosphatase activities of *Micrococcus pyogenes*. J. Bacteriol. 73:100–104.
2. Blair, J. E., and M. Carr. 1953. The bacteriophage typing of staphylococci. J. Infec. Dis. 93:1–13.
3. Christie, R., N. E. Atkins, and E. Muench-Petersen. 1944. A note on a lytic phenomenon shown by the group B streptococci. Aust. J. Exp Biol. Med. Sci. 22:197–200.
4. Hunt, G. A., and A. J. Moses. 1958. Acute infection of mice with Smith strain of *Staphylococcus aureus*. Science 128: 1574–1575.
5. Iwata, K., and T. Eda. 1968. Studies on unique staphylococcal strains exhibiting high virulence for mice by intraperitoneal injection. I. Morphological and serological properties of the strains. Jap. J. Bacteriol. 23:165–171.
6. Jungerman, D., and D. Lipnicki. 1969. Investigations of the immunological properties of a coagulase-negative mutant of *Staphylococcus aureus*. Bull. Acad. Polanaise Sci. 17:101–107.
7. Kamijo, K., and S. Inoue. 1969. Studies on the virulence of staphylococci. II. Activities of extracellular enzymes in culture filtrates. Jap. J. Bacteriol. 24:278–283.
8. Koenig, M. G. 1962. Factors relating to the virulence of staphylococci. I. Comparative studies on two colonial variants. Yale J. Biol. Med. 34:537–559.
9. Rogers, D. E. 1966. Experimental observations on staphylococcal disease, p. 279–296. *In* Symposium on staphylococci and staphylococcal infections. Panstwowe Wydawnictwo Naukowe, Warszawa.
10. Smith, A. M., and W. B. Wood. 1956. An experimental analysis of the curative action of penicillin in acute bacterial infections. J. Exp. Med. 103:499–507.
11. Wiley, B. B. 1969. Capsule size, coagulase production, and egg virulence among certain strains of *Staphylococcus aureus*. Can. J. Microbiol. 14:685–689.
12. Yoshida, K. 1962. On the change of the virulence and its related factors with drug resistant mutants of strains of *Staphylococcus aureus*. New Med. J. 48:463–467.
13. Yoshida, K., and R. D. Ekstedt. 1968. Relation of mucoid growth of *Staphylococcus aureus* to clumping factor reaction, morphology in serum-soft agar, and virulence. J. Bacteriol. 96:902–908.
14. Yoshida, K., and M. Takahashi. 1962. On the pigment production of *Staphylococcus aureus* related to drug resistant mutants. Medicine 19:657–658.
15. Yoshida, K., M. Takahashi, and T. Takeuchi. 1969. Pseudocompact-type growth and conversion of growth types of strains of *Staphylococcus aureus* in vitro and in vivo. J. Bacteriol. 100:162–166.