INFECTION AND IMMUNITY, July 1977, p. 231-234

Vol. 17, No. 1
Printed in U.S.A.

NOTES

Nature of the Genetic Determinant Controlling Encapsulation in *Staphylococcus aureus* Smith

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Received for publication 24 January 1977

Two strains of *Staphylococcus aureus*, Smith and M, were studied for the elimination of encapsulation. For *S. aureus* M, encapsulation was stable. For *S. aureus* Smith, spontaneous loss of encapsulation was 1.3% and increased markedly in medium containing surface-active agents. In the presence of sodium dodecyl sulfate, unencapsulated cells had a considerable selective advantage. Attempts to demonstrate covalently closed circular plasmid deoxyribonucleic acid were unsuccessful. In cultures of unencapsulated cells, encapsulated cells were observed occasionally. These data argue against a plasmid location for the determinants controlling encapsulation in this organism in spite of a high spontaneous loss of this character.

The Smith strain of *Staphylococcus aureus* is the prototype of an encapsulated staphylococcus highly virulent for mice. When grown in laboratory media, the occurrence of two colonial variants is readily observed. The diffuse variant is encapsulated and highly virulent for mice, whereas the compact unencapsulated variant is relatively avirulent (2, 4). Although the polysaccharide capsular antigen has been characterized, little is known regarding the genetics of encapsulation in this organism. The ease with which unencapsulated variants can be obtained from encapsulated organisms (2, 4) indicated that the genetic determinants controlling encapsulation in *S. aureus* Smith may be located on a plasmid. In a preliminary study (done by J.T.P. and L.V.) which suggested that loss of the encapsulation character was irreversible, approximately 5×10^4 colonies were screened from a 24-h culture of the unencapsulated Smith strain, and none was produced by encapsulated cells. In another study (done by J.T.P.), from a 2-week-old culture of the unencapsulated Smith strain, colonies of encapsulated cells were observed. The purpose of this investigation was to resolve these apparently conflicting results and to determine whether encapsulation in *S. aureus* Smith and another well-characterized encapsulated staphylococcus, *S. aureus* M (6), is controlled by chromosomal or extrachromosomal determinants.

The Smith encapsulated strain of *S. aureus* has been maintained in our laboratory for several years and was obtained originally from the Sharp and Dohme Division of the Merck Institute for Therapeutic Research. The encapsulated strain is not phage typable with phages from the International Series, whereas unencapsulated variants are lysed by the routine test dilution (RTD) of *S. aureus* phage 44A. The M encapsulated strain of *S. aureus* was kindly provided by M. A. Melly, Vanderbilt University School of Medicine.

Spontaneous loss of capsule production was determined by inoculating a single colony of the Smith encapsulated strain into Trypticase soy broth (BBL) containing Difco yeast extract (0.3%, wt/vol) (YETS). After incubation overnight at 37°C, the culture was used to inoculate YETS broth (10^7 cells per ml of medium) which was incubated with shaking for 18 h at 37°C. To test for elimination by growth at elevated temperature or upon storage, cells were incubated in YETS broth for 18 h at 44°C with shaking or for 3 weeks at 37°C, respectively. To test for elimination of encapsulation with ethidium bromide (Calbiochem, La Jolla, Calif.), cells were incubated with shaking for 18 h at 37°C in YETS broth containing 5×10^{-6}, 1×10^{-6}, or 5×10^{-7} M ethidium bromide. To test for elimination with sodium dodecyl sulfate (SDS; Sigma Chemical Co., St. Louis, Mo.), tetradecyl sulfate (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N.Y.), or sodium

deoxycholate (Difco), cells were incubated in YETS broth containing 0.002, 0.0025, or 0.003% (wt/vol) SDS, 0.00025, 0.0005, 0.001, or 0.002% (wt/vol) tetradecyl sulfate, or 0.032% (wt/vol) sodium deoxycholate for 18 h at 37°C with shaking.

To determine the presence of unencapsulated cells in all cultures, dilutions were made in distilled water and samples were plated on brain heart infusion (BHI; Difco) agar so as to yield approximately 400 to 500 colonies per plate. After incubation for 2 days at 37°C, a molten mixture of safranine (0.02%, wt/vol) and agar (0.8%, wt/vol) was poured over the surfaces of the BHI agar plates. After 2 days at room temperature, colonies produced by unencapsulated cells stained red, whereas colonies produced by encapsulated cells stained pink. Cells from red colonies were also inoculated into YETS broth, incubated overnight at 37°C, and then typed with phage 44A. Unencapsulated cells are lysed at the RTD, whereas encapsulated cells are resistant to lysis. When these methods for detection of unencapsulated cells were compared with negative staining with India ink and observation by microscopy, they were accurate and dependable substitutes. As a result of the ease in screening large numbers of cells by our overlay and staining procedure and subsequent testing of a representative number (1 to 10%) of cells identified as unencapsulated, a reliable method for their detection was available.

The elimination of encapsulation spontaneously, at elevated temperature (44°C), upon storage for 3 weeks at 37°C, or in the presence of various curing agents for *S. aureus* Smith is shown in Table 1. The spontaneous loss of encapsulation was 1.3%, and the loss of encapsulation at 44°C, upon storage, or in the presence of ethidium bromide varied from 1.0 to 1.8%. Growth in the presence of SDS, tetradecyl sulfate, or sodium deoxycholate resulted in an enhancement of the loss of encapsulation, the most consistent rates of loss occurring with SDS.

The M encapsulated strain was tested for spontaneous loss of encapsulation and loss by incubation in YETS broth or YETS broth containing 0.003% (wt/vol) SDS. A total of 19,087 colonies was screened for the spontaneous elimination of encapsulation daily over a 7-day period, and only 2 (0.01%) colonies produced by unencapsulated cells were observed. Growth in the presence of 0.003% SDS resulted in the scoring of 1 (0.003%) colony formed by unencapsulated cells among 37,172 colonies screened.

To determine whether encapsulated cells were losing this character or whether unencapsulated cells were being selected, growth from a colony of the Smith encapsulated strain was inoculated into YETS broth and incubated overnight at 37°C. The culture was then diluted in distilled water so that 10^7 encapsulated cells per ml of medium were inoculated into YETS broth containing 0.002% SDS and incubated with shaking for 18 h at 37°C. The culture was sampled hourly, and appropriate dilutions in distilled water were spread on BHI agar plates. Plating, screening, and scoring of colonies were the same as described previously. Figure 1 shows the appearance of unencapsulated cells

TABLE 1. *Elimination of encapsulation under various growth conditions or with various curing agents for S. aureus Smith*

Type of growth condition or curing agent	Concn of curing agent	No. of colonies screened	No. of unencapsulated colonies	Percent elimination
Spontaneous		205,195	2,653	1.3
Elevated temperature (44°C)		34,166	502	1.5
Storage (3 wk at 37°C)		9,472	96	1.0
Ethidium bromide	5×10^{-6} M	11,357	203	1.8
	1×10^{-6} M	4,447	47	1.1
	5×10^{-7} M	13,878	253	1.8
Sodium dodecyl sulfate	0.002%	13,793	5,682	41.2
	0.0025%	1,026	457	44.5
	0.003%	2,213	1,005	45.4
Tetradecyl sulfate	0.00025%	3,064	16	0.5
	0.0005%	1,802	51	2.8
	0.001%	8,667	6,257	72.2
	0.002%	10,046	1,475	14.7
Sodium deoxycholate	0.032%	16,552	1,329	8.0

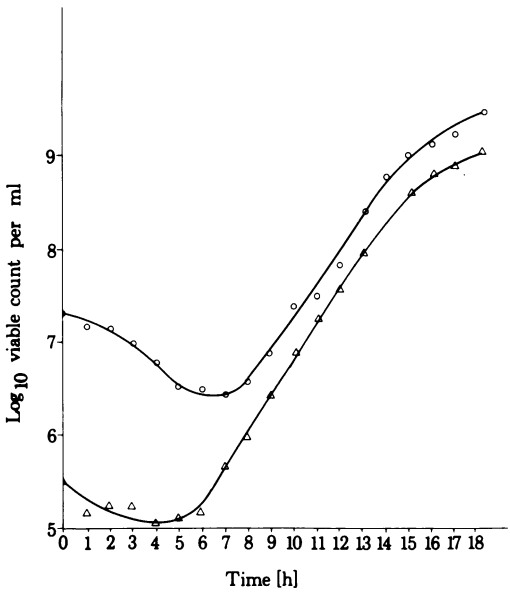

FIG. 1. *Appearance of unencapsulated cells during growth of S. aureus Smith in YETS broth containing 0.002% SDS.* (○) *Total cells;* (△) *unencapsulated cells.*

during growth of *S. aureus* Smith in YETS broth containing 0.002% SDS. The total number of viable cells decreased 1 log after 6.5 h; then the number of cells increased and was logarithmic by 8 h. Unencapsulated cells in the culture decreased 0.5 log by 4.5 h, but growth was logarithmic in less than 6 h. The logarithmic rate of growth of the encapsulated and unencapsulated cells was the same, and both types reached the stationary phase at the same time.

To determine whether loss of encapsulation was reversible, a single colony of the Smith unencapsulated strain was inoculated into YETS broth and incubated for 24 h at 37°C. Dilutions were then made in distilled water, samples were plated on BHI agar plates so as to yield approximately 200 colonies per plate, and plates were incubated for 2 days at 37°C. Screening and scoring of colonies were as described previously. In another study, a single colony of the Smith unencapsulated strain was inoculated into YETS broth and incubated for 2 weeks at 37°C. At 2-day intervals, an inoculum was streaked onto a BHI agar plate so as to yield approximately 100 isolated colonies. After incubation for 2 days at 37°C, plates were observed for colonies produced by encapsulated cells. None of approximately 5×10^4 colonies grown from a 24-h culture of the unencapsulated Smith strain was produced by encapsulated cells. In the culture of the same organism incubated for 2 weeks and sampled at 2-day intervals, from a total of approximately 700 colonies screened, a single mucoid colony, whose cells were subsequently resistant to lysis by phage 44A, was observed. Subsequent isolation of unencapsulated cells arising spontaneously from this encapsulated revertant and their susceptibility to lysis by phage 44A indicated that the encapsulated revertant resembled the original encapsulated parental organism.

Deoxyribonucleic acid (DNA) from encapsulated and unencapsulated strains was labeled with tritiated thymidine, cells were lysed with lysostaphin, and the lysates were centrifuged in CsCl-ethidium bromide gradients as described previously (7). Analysis of the CsCl-ethidium bromide gradients of DNA isolated from the encapsulated and unencapsulated strains yielded identical profiles. A single, prominent band corresponding to chromosomal DNA was detected. No satellite plasmid bands were observed. Plasmid DNA was isolated from a strain of *S. aureus* containing a penicillinase plasmid used as a positive control.

Instability of a phenotypic characteristic usually suggests that the relevant determinants may be located on a plasmid. As shown in Table 1, the spontaneous loss of encapsulation in *S. aureus* Smith is high enough to suggest a plasmid location, since a frequency of loss of 1 in 10^3 to 10^5 per cell division is characteristic of many plasmid markers (5). Neither incubation at 44°C, storage at 37°C for 3 weeks, nor growth in the presence of ethidium bromide enhanced the spontaneous loss of encapsulation, although these have been effective procedures to eliminate other plasmid-located determinants in *S. aureus* (1, 3). The marked increase in the appearance of unencapsulated cells when grown in the presence of the three anionic surface-active agents, SDS, tetradecyl sulfate, and sodium deoxycholate supports the effects of these chemical agents as curing agents for other extrachromosomal determinants in this organism (8). However, these data are valid only if it can be shown that unencapsulated cells are produced and not selected during treatment of the encapsulated culture. As shown in Fig. 1, the rapid increase of unencapsulated cells in the culture of originally encapsulated cells after 4.5 h is due to the marked selective advantage they have when grown in the presence of SDS. Chou and Sonstein (Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, H33, p. 101) reported recently that SDS causes a loss in the ability to produce penicillinase in *S. aureus* both by the selective killing of penicillinase-producing cells and by the elimination of the penicillinase plasmid

from the survivors. The latter mechanism was the principal cause for the appearance of penicillinase-negative cells. The unsuccessful attempts to demonstrate covalently closed circular plasmid DNA by ethidium bromide gradient centrifugation also argue against a plasmid site for the genes coding for the production of a capsule, in spite of a rather high (1.3%) spontaneous loss of this character. According to Lacey (5), irreversibility of a change in the absence of introduction of new genes by a transfer process and the loss of two or more phenotypic characters simultaneously are indicative of a plasmid. The isolation of even a rare encapsulated organism from a culture of unencapsulated cells, as we observed in this study and several other times in working with this organism and as was also reported by Koenig (4), argues strongly against a plasmid-located determinant(s).

The stability of encapsulation in *S. aureus* M and the inability of SDS to affect this character point clearly to a chromosomal location for the encapsulation determinant(s) in this organism. Obviously, there are differences between the genetic determinants of encapsulation in *S. aureus* Smith and *S. aureus* M.

This investigation was supported by a grant from the University of Missouri-Columbia Research Council and by Public Health Service grant AI-08450 from the National Institute of Allergy and Infectious Diseases.

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