Protein A Mutants of Staphylococcus aureus

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Staphylococcus aureus Cowan I was exposed to nitrosoquaiidine or ethylmethanesulfonate, and survivors were screened on nutrient agar plates containing rabbit anti-protein A serum for loss of protein A production. More than half of all protein A-deficient mutants also lacked nuclease, coagulase, alpha hemolysin, fibrinolysin, mannotol utilization, and the phage-type pattern. Mutants with a spectrum of these properties were also isolated. Induced or spontaneous reversions of the mutants were observed. The properties of the protein A-deficient mutants suggest that synthesis or release (or both) of a number of extracellular products of S. aureus is controlled by a common regulatory mechanism.

Protein A has been shown to be associated with the cell surface of most Staphylococcus aureus strains (13). In addition, protein A can be detected as an extracellular product in the culture medium of bacterial and L-forms of S. aureus (12). A direct reaction between protein A and the Fc part of gamma G globulins from many mammalian species can be demonstrated (11, 14, 15, 23). Cell-bound protein A has been heat-extracted, purified, and characterized (16). The molecular weight for such preparations has been calculated to be 15,000. Amino acids accounted for approximately 90% of the preparation, and all the common amino acids except tryptophan and cystine were detected.

In the present study, protein A-deficient mutants of S. aureus were isolated and characterized with respect to cell-bound and extracellular protein A, nuclease, coagulase, alpha hemolysin, fibrinolysin, utilization of mannitol, and phage type.

MATERIALS AND METHODS

Strain. S. aureus strain Cowan I, phage type 52, was used. The strain has a high content of protein A as previously described (K. Jensen, Thesis, Munksgaard, Copenhagen, 1959).

Substrate and culture. For toxin production, a semisynthetic medium prepared as described by Casman (5) was used with the addition of 2% protein hydrolysate (3). To obtain a solid medium, 1.5% agar (Difco) was used. The staphylococci were cultured on a solid medium covered with cellophane (17).

Assays of enzymes and toxins. Coagulase formation was determined by using Coagulase Plasma (Difco) exactly according to the instructions provided by the manufacturer (9). Nuclease activity was demonstrated on DNase Test Agar (BBL; reference 7). All isolated strains were tested for mannitol utilization on agar plates containing phenol red plus mannitol.

The alpha-hemolysin titer was measured by incubating 0.5 ml of serial twofold dilutions of the sample to be investigated with 0.5 ml of a 1% suspension of washed rabbit erythrocytes for 1 hr at 37 C and 1 hr at room temperature (24).

Staphylokinase (fibrinolysin) was detected on agar plates composed of 15 parts of rabbit plasma and 85 parts of heart infusion agar. The plates were prepared by using matrices for the basin plate technique. The basins were filled with the samples to be tested, and the plates were incubated at 37 C for 24 hr (6).

Phage typing. The technique described by Blair and Williams (4) was used with the standard phage set.

Sensitivity tests. The disc diffusion method described by Ericsson (10) was used for antibiotic sensitivity tests. Ordinary sheep blood-agar plates were used.

Serum. Antisera against protein A were produced by immunizing albino rabbits (2 to 2.5 kg) with highly purified protein A. Two subcutaneous injections of 0.25 mg of protein A in Freund's adjuvant were given 3 weeks apart. Blood was collected 2 weeks after the last injection (15).

Determination of protein A. Bacteria were grown in tubes containing 5 ml of nutrient broth to a concentration of 10⁶ colony-forming units per ml and were harvested by centrifugation. The content of extracellular protein A in the supernatant fluid and the cell-bound protein A, quantitatively extracted from the bacteria by the method of Jensen (Thesis, Munksgaard, Copenhagen, 1959), were determined separately. The hemagglutination technique for quantitation of protein A (32) was used, beginning with a dilution of 1:1, followed by serial twofold dilutions. The protein A titers given are the values obtained for extracellular protein A produced in a broth culture (10⁶ cells per ml).

Precipitation in gel (15) was performed against rabbit anti-protein A serum in 1% agar (Special Agar Noble, Difco) in 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.2).

Detection of protein A-deficient (SpA-) mutants or protein A-producing (SpA+) revertants. Broth cultures were spread on 1.5% nutrient agar plates with an
overlay of 0.7% nutrient agar containing rabbit anti-protein A serum or immunoglobulin G prepared from such serum. A precipitation ring appeared around protein A-producing colonies after the agar plates had been incubated for 24 hr at 37 C and 24 hr at 4 C.

Selection of mannitol-utilizing revertants. Cultures were spread on eosin-methylene blue (EMB) agar plates (29). The medium consisted of (per liter): Peptone Peptone (Difco), 10 g; yeast extract, 1 g; K2HPO4, 2 g; d-mannitol, 10 g; methylene blue chloride, 1.75 mg; eosin Y, 8.0 mg; and agar, 15 g. The plates were incubated for 48 to 72 hr at 37 C. Colonies of mannitol-utilizing bacteria were red.

Treatment with acriflavine. A 0.2- to 0.5-mI amount of a 4-hr culture of S. aureus Cowan I was inoculated into fresh medium containing acriflavine in concentrations of 5, 12.5, or 25 μg/ml (28). The incubation time was 48 hr at 37 C.

Mutagenesis. To a suspension of washed cells from an overnight culture, freshly dissolved N-methyl-N'-nitro-N-nitrosoguanidine (NG; references 1, 2) was added to a final concentration of 100 μg/ml. After incubation at 37 C for 60 min, the cells were washed and suspended in 5 ml of nutrient broth, incubated for 6 hr at 37 C to allow phenotypic expression, and then analyzed.

A 2-ml amount of an overnight culture was carefully mixed with 0.4 ml of ethylmethane-sulfonate (EMS; reference 25) and incubated for 10 min at 37 C. The cells were washed in saline, resuspended in 5 ml of nutrient broth, and incubated for 6 hr at 37 C.

Production of revertants. The mutants obtained with NG were treated with 0.04 ml of EMS per 2 ml of overnight culture for 30 min (31). The EMS mutants were treated with NG as above.

RESULTS

Screening technique for protein A production. Protein A has been shown to interact with the Fc fragment of normal human gamma G globulin (14). When S. aureus Cowan I was grown on nutrient agar containing normal human gamma G globulin in a concentration of 0.05 mg/ml, weak and diffuse precipitation halos appeared around protein A-producing colonies. When a higher or lower concentration of normal human gamma G globulin was used, the precipitation ring was more diffuse or could not be detected. S. aureus Cowan I was therefore grown on agar plates containing different concentrations of anti-protein A gamma G globulin from rabbit. Figure 1a shows the readily visible and sharp precipitation zones obtained with anti-protein A rabbit gamma G globulin in a concentration of 0.1 mg/ml. Corresponding results were obtained with rabbit antiserum. The best results were obtained with an incubation time of 24 hr at 37 C followed by 24 hr at 4 C. Figure 1b shows that no precipitation ring could be detected around bacterial colonies not producing protein A.

Isolation of protein A-deficient mutants. In all, 60 protein A-deficient mutants were isolated on agar containing anti-protein A immunoglobulin G after treatment with NG or EMS. All mutants were grown in nutrient broth and the contents of cell-bound and extracellular protein A were determined by the hemagglutination technique and by precipitation in agar gel against anti-protein A from rabbits. Protein A-producing control colonies from the same plates were investigated for protein A production in the same way. About 0.1% of all fully grown colonies examined were mutant colonies. In control experiments with nonmutagenized bacteria, all of about 40,000 colonies produced protein A.

Extrachromosomal location of determinants for antibiotic resistance and toxin production has been described (8, 20, 28, 30). Treatment with acriflavine has resulted in high frequencies of elimination of these properties (28). Doses of acriflavine higher than 5 μg/ml were lethal for S. aureus Cowan I. Approximately 5,000 colonies were examined after treatment with acriflavine in a concentration of 5 μg/ml, and only one protein A-deficient mutant was found. Untreated bacteria incubated at 42 C instead of 37 C showed no loss of protein A production, as might have occurred if elimination of a plasmid was responsible for the appearance of protein A-deficient mutants (27). More than 30,000 colonies were examined.

Characteristics of protein A-deficient mutants. Protein A-deficient mutants were assayed for the following activities: nuclease, coagulase, alpha hemolysin, fibrinolysin, and mannitol utilization. The mutants were not tested for beta and delta hemolysin, because the wild type produced low or not detectable amounts of these toxins when tested by standard methods (18, 24). The phage type pattern of the mutants was also investigated. Table 1 shows six different classes of protein A-deficient mutants obtained with NG. Class I NG (10 mutants) showed a reduced production of cell-bound and extracellular protein A. Protein A production was reduced from ½6 to ½4 of the original as measured with the hemagglutination technique. However, the other properties investigated were intact. Class II NG includes four mutant strains which showed only a total lack of protein A production. However, the mutants in both class I and II showed a reduction in alpha hemolysin production. In almost all these cases, there appeared to be a correlation between the reductions in alpha hemolysin and protein A titers. Class III NG (1 mutant) showed a reduced production of protein A and also a deficiency in coagulase and alpha hemolysin production. One mutant, class IV NG, was negative for all properties except coagulase production and mannitol utilization. The mutants in class V NG (2 mutants) were deficient in all properties except nuclease
production. More than a third of all mutants obtained with NG (10 mutants, class \( V_{NG} \)) were negative for all the activities investigated. The phage-type pattern changed from 52/52A/80/81 to not typable.

Table 2 shows the different classes of protein A-deficient mutants obtained with EMS. The distribution in classes is similar to that obtained with NG. The mutant in class I\(_{EMS}\) (2 mutants), V\(_{EMS}\) (2 mutants), and V\(_{EMS}\) (24 mutants) have the same patterns as those in I\(_{NG}\), V\(_{NG}\), and V\(_{NG}\), respectively. Class II\(_{EMS}\) (1 mutant) showed a reduced protein A production and deficiency in alpha hemolysin and mannitol utilization. One mutant, class III\(_{EMS}\), was negative for all properties except mannitol utilization. Class IV\(_{EMS}\) mutants (2 mutants) were deficient for all properties except coagulase. A mutant obtained with acriflavine is also included in Table 2. It had the same pattern as III\(_{EMS}\). All mutants obtained were shown to have the same antibiogram as the wild type, Cowan I.

**Spontaneous reversion of protein A-deficient mutants.** Some mutants reverted spontaneously at high frequency, especially from classes I\(_{NG}\) and I\(_{EMS}\), whereas no spontaneous reversion was found for other classes when tested for protein A production, although more than 5,000 colonies were investigated. The frequency of spontaneous reversion for classes V\(_{NG}\) (NG 223) and V\(_{EMS}\) (EMS 312) was also determined by selection on EMB agar containing mannitol. As shown in Table 3, the frequency was \(10^{-8}\) to \(10^{-7}\). However, all revertants obtained from either mutant (NG 223 and EMS 312) had a deficient coagulase production. The highest frequency of spontaneous reversions was observed in class I\(_{NG}\) mutants. When one strain, NG2, class I\(_{NG}\), reverted spontaneously or after treatment with mutagen, a spectrum of protein A production was observed in the segregants in high frequency. When mutant NG 357, class I\(_{NG}\), was assayed for spontaneous reversions, segregants of the class VI type lacking all the tested extracellular activities and phage type were observed as well as wild-type segregants. The result indicates that the pheno-
typic expression of all the tested activities may be controlled by a single locus.

**Induced reversion of protein A-deficient mutants.** Attempts to revert NG mutants with EMS and EMS mutants with NG were undertaken to characterize the combined loss of cell-bound as well as extracellular protein A, nuclease, coagulase, alpha hemolysin, fibrinolysin, utilization of mannitol, and change in phage type. The mu-

![Fig. 1. Nutrient agar plates containing rabbit anti-protein A serum with (a) colonies of Staphylococcus aureus Cowan I producing protein A and (b) colonies of mutants not producing protein A.](image-url)
Revertants selected for induced reversion had a spontaneous reversion frequency of $10^{-4}$ or less.

Revertants were isolated from at least two mutants in the classes indicated in Table 3. Revertants were selected for mannitol utilization on EMB agar plates and for protein A production on nutrient agar plates containing rabbit anti-protein A serum. Table 3 shows some typical experiments in which revertants were analyzed.

When treating class $I_{NG}$ mutants with EMS, the wild-type property of high protein A production was regained with a frequency of 0.1%. However, revertants with a spectrum of low protein A production were also obtained. In class $VI_{NG}$ (Table 3) the revertants had a reduced production of protein A, but in most cases other properties were regained. Utilization of mannitol in NG 53 and production of coagulase in NG 223 were, however, not restored. After NG treatment of the class $IV_{EMS}$ mutant (EMS 6) and selection for mannitol utilization, revertants which regained all wild-type characters were obtained (Table 3). All properties reverted en bloc when a class $VI_{EMS}$ mutant was treated with NG. However, the production of protein A by the revertant was reduced.

**DISCUSSION**

Tables 1 and 2 show the phenotypic patterns of protein A-deficient strains. All strains except the leaky mutants lacked cell-bound as well as extracellular protein A as determined with an anti-protein A serum. The site of mutation is uncertain, but termination, frameshift, or deletion of the structural gene for protein A as well as alterations in hypothetical regulatory genes are all possible.

Classes $I_{NG}$, $II_{NG}$, $I_{EMS}$, and $II_{EMS}$ represent mutants which exhibit loss or deficiency only in protein A production. However, there was also a reduction in alpha hemolysin production by most mutants in both classes, and the reduction in protein A production appeared to be correlated to the alpha hemolysin titer. All other extracellular activities were determined only with qualitative tests. Thus, loss, but not reduction, in activity could be observed.

Classes $III_{NG}$ to $VI_{NG}$ and $III_{EMS}$ to $VI_{EMS}$ exhibited a spectrum of deficiencies of protein A, nuclease, coagulase, alpha hemolysin, fibrinolysin, and phage type in different combinations. More than half of all mutants (classes $VI_{NG}$ and $VI_{EMS}$) showed a deficiency in all properties investigated. Multiple mutations, although not excluded, are improbable, as NG treatment of the wild type gave mutation frequencies of 0.2 to 0.5% for isolated loss of nuclease or of mannitol utilization. The return of all properties in reversion experiments (Table 3) suggests a common control mechanism for the phenotypic expression of these properties. In many cases one or two of the properties were not regained en bloc. This was true for protein A, coagulase, mannitol utilization, and phage type pattern, indicating the possibility of double mutants in these cases.

Results that may be complementary to these findings in regard to protein A-deficient mutants have been published. Korman (22) reported mutants characterized by their failure to elaborate coagulase, their resistance to bacteriophage, and their ability to use mannitol. McClatchy and Rosenblum (26) reported that 11 of 20 alpha hemolysin-negative mutants also lacked fibrinolysin and that both groups could be transduced to wild-type characters.

In studies of penicillinase production, Harmon and Baldwin (19) noted a mutant which failed to produce detectable amounts of alpha hemolysin, fibrinolysin, lipase, gelatinase, and caseinase, but which had normal nuclease, coagulase, and beta hemolysin activities. These findings may indicate that two or more sets of genes for extracellular enzymes are under coordinated, but separate, control. Omenn and Friedman (31) reported
gained together.

all experiments, S. aureus.

than production coagulase, and (unpublished data).

to glutination and staphylococcal results, no by clinical strains.

of Omenn and Friedman alteration detected was leaky mutants which had a combined loss of nuclease, coagulase, and beta hemolysis. In reversion experiments, all three enzyme functions were regained together. However, in contrast to our results, no coordination of alpha hemolysin production and phage type could be shown. Forsgren (13) reported a good correlation between production of coagulase, nuclease, and protein A by clinical strains. All of 700 coagulase-positive staphylococcal strains isolated from humans produced nuclease, and 692 produced protein A.

Very little is known about the regulation of synthesis and release of extracellular products of S. aureus. No cell-bound or intracellular protein A could be detected in the mutants, other than leaky mutants in which intracellular protein A was detected at reduced levels but in proportion to secreted protein A. A leaky (NG 70, class I\textsubscript{NG}) mutant was shown to produce less protein A than the wild type. However, protein A from the mutant and the wild type had the same specific immunological activity as determined by hemagglutination (unpublished data). This observation may suggest a regulatory rather than a structural alteration in the mutant. Although induction and product inhibition have not been demonstrated, it is possible that regulation is subject to coordinated induction so that an inducer of one enzyme leads to the production of several as suggested by Ommen and Friedman 1970. The properties of the protein A-deficient mutants point to coordinated control of several extracellular products and phage type. Further information on the genetic relatedness of these properties will be obtained by transduction experiments now in progress.

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**LITERATURE CITED**


