Isolation of Mutants of *Staphylococcus aureus* Lacking Extracellular Nuclease Activity

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Suspensions of *Staphylococcus aureus* Fogli strain were exposed to nitroso-guanidine and screened on deoxyribonucleic acid-acridine orange-agar plates for loss of extracellular nuclease activity. All nuclease-deficient mutants lacked cross-reacting material when tested with antinuclease antibody. Coagulase and \( \beta \)-hemolysin were also lost in nuclease-deficient mutants, and all three enzyme functions were regained together upon reversion with ethyl methane sulfonate. These observations are consistent with the suggestion that the synthesis or the release, or both the synthesis and release, of certain extracellular enzymes may be subject to coordinated control mechanisms.

*Staphylococcus aureus* secretes into its growth medium or site of infection a variety of potent degradative enzymes, including coagulase, several hemolysins, hyaluronidase, and nuclease. Studies over the past 6 years have characterized, in great detail, the extracellular nuclease of the Fogli strain of *S. aureus* (6, 19). This enzyme hydrolyzes either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) and consists of a single polypeptide chain of 149 amino acid residues. The complete amino acid sequence has been reported (8, 19). Certain residues have been implicated in the active site, and chemical modification of a single tyrosine residue (position 85) completely destroys enzyme function (5). On the other hand, modification of individual residues in several types of chemical derivatives does not prevent precipitation with antinuclease antibody (9; G. S. Omenn, D. A. Ontjes, and C. B. Anfinsen, 1970, Biochemistry 9: 304–312). Thus, enzymatically inactive, antigenically cross-reacting derivatives of nuclease are expected among mutations that cause single amino acid substitutions.

This paper reports on the isolation and characterization of nuclease-deficient mutants of *S. aureus*.

**MATERIALS AND METHODS**

The Fogli strain of *S. aureus* (16) was used for all studies. It is a penicillin-sensitive producer of nuclease, coagulase, \( \alpha \)-hemolysin, and \( \beta \)-hemolysin, its phage type is 53/83, and it possesses typical susceptibility to lysis by lysostaphin (11).

**Mutagenesis.** Overnight cultures were centrifuged, and the washed cells were suspended in phosphate buffer, pH 7.0. Freshly dissolved, membrane filter (Millipore Corp.)-sterilized \( N \)-methyl-\( N' \)-nitro-\( N \)-nitroso-guanidine (NG; references 1, 2) was added to give a final concentration of 100 \( \mu \)g/ml, and the suspensions were incubated at 37°C for 30 min. Samples were diluted 50 times in nutrient broth and incubated overnight; then dimethylsulfoxide (10\%, v/v) was added to enhance viability upon freezing and thawing, the titer was determined, and suspensions were frozen for subsequent plating.

Screening technique for detection of nuclease-deficient (nuca-) mutants. The method of Lanyi and Lederberg (12) for the detection of *Bacillus subtilis* ribonuclease activity was adapted to the preparation of DNA-acridine orange (AO)-agar, with the addition of 40 \( \mu \)g of AO per ml to Difco DNotase Test Agar before autoclaving. The resulting medium gave brilliant yellow-green fluorescence. Release and diffusion of nuclease from a colony led to a dark halo of defluorescence, as the enzyme cleaved DNA to oligonucleotides and the agar served to quench free AO. Fluorescence persisted around a nuca- colony.

**Characterization of mutants.** Presumptive nuca- colonies were streaked on DNA-AO-agar, inoculated into rich, liquid medium containing Casamino Acids, yeast hydrolysate, lactic acid, and salts (17), and incubated overnight. Overnight growth was estimated by turbidity at 700 nm. Quantitative nuclease activity was assayed in a Gilford spectrophotometer with DNA as the substrate (4). Supernatant fluid was tested for cross-reacting material (CRM) by precipitation with antinuclease antibody (G. S. Omenn, D. A. Ontjes, and C. B. Anfinsen, Nature, in press) in Hy-
land agar immunodiffusion plates. Culture supernatant fluid was assayed for coagulase by inoculation into a solution of rabbit plasma diluted 1:7 with sterile saline and incubated, together with controls, for 4 to 24 hr at 37 C. Production and release of α- and β-hemolysins were identified by formation of clear zones of hemolysis on rabbit and sheep blood-agar, respectively. Susceptibility to lysis with lysostaphin (kindly provided by Peter Tavormina of Mead Johnson Research Center, Evansville, Ind.) was measured by a decrease in turbidity at 700 nm of a suspension of cells incubated at room temperature with 1 unit of lysostaphin per ml of medium. Phage typing with standard typing phages was performed by Charles Zierdt.

Production of revertants. Reversion experiments were carried out by adding 40 μl of a solution of ethyl methane sulfonate (EMS; 19 mg/ml) to 2 ml of *S. aureus* nuc− mutant cells resuspended in phosphate buffer. After incubation at 37 C for 30 min, dilution of the mutagen in broth, incubation of samples, and determination of titer, colonies were screened on either sheep blood-agar or on DNA-AO-agar for revertants to normal β-hemolysin or nuclease activity.

**RESULTS**

In all, 40 nuclease-deficient mutants were isolated by this procedure. All were confirmed by streaking on DNA-AO-agar plates and by subsequent quantitative assay of liquid culture supernatant fluids. Nuclease-containing control colonies from the same plates were streaked and assayed along with each suspected nuc− colony. Mutants represented about 0.1% of all fully grown colonies examined. AO, in the absence of NG as a mutagen, produced no nuclease-deficient colonies in two types of experiments. First, nonmutagenized suspensions of *S. aureus* were plated on DNA-AO-agar. Second, such suspensions were plated on DNase Test Agar without AO and then were replica-plated onto DNA-AO-agar. All replicated colonies grew normally and were nuc+. Approximately 20,000 colonies were examined in these control experiments. Replica-plated colonies incubated at 42 C instead of 37 C also had no loss of nuclease production, as might have occurred if elimination of a plasmid were responsible for the appearance of the nuc− colonies (16).

Wild-type cultures contained 4 to 10 μg of nuclease per ml of supernatant fluid, by spectrophotometric assay and by quantitative binding of nuclease to an affinity chromatography column, in which the inhibitor deoxymethylidine-3′-5′-diphosphate was coupled to Sepharose (7). Antinuclease antibody could detect nuclease in concentrations of about 10 μg/ml in the agar immunodiffusion test. With two- to fourfold concentrations of supernatant fluids before reaction with antiserum, all nuc+ cultures gave sharp precipitation lines. To increase sensitivity so as to permit the detection of antigenic activity corresponding to 10% of the normal amount of wild-type nuclease, mutant culture supernatant fluids were concentrated 20 times by lyophilization. All 40 nuc− mutants were found to be CRM− in these tests. Thus, no enzymatically deficient, antigenically intact mutants were obtained.

Nuclease-deficient mutants were assayed for three other extracellular enzymes. Only one mutant had an isolated deficiency (CRM−) in nuclease (Table 1, class I). The others had lost both coagulase and β-hemolysin (class II and class III). These strains also grew poorly on tellurate-glycine-agar, a medium which inhibits growth of non-coagulase-producers. By contrast, α-hemolysin activity was normal in all of the mutants.

Among the nuclease-deficient, coagulase-deficient, β-hemolysin-deficient, α-hemolysin-containing mutants, three of six strains tested showed alteration in two characteristics of the cell surface. Whereas the parent Foggi strain had phage type 53/83, these mutants (class III) were untypeable by the standard phages. Suspensions of these mutants were lysed less than 10% in 30 min by lysostaphin, whereas the parent strain and other mutants were lysed 80% or more within 10 min at room temperature with 1 unit of lysostaphin per ml. With 5 units of lysostaphin per ml for several hours, gradual lysis did occur.

The possibility that nuclease and other enzymes were being synthesized, but not being released or secreted through the cell surface, was investigated in mutants of each of the three classes (Table 1). Complete lysis with 5 units of lysostaphin per ml, sonic treatment for a total of 3 min, or treatment with a French press each released intracellular contents from washed cells, as judged by the reaction of Lowry et al. (13) for protein and by the lack of intact staphylococci on Gram stain examination. Nevertheless, no nuclease activity could be detected in lysates of the mutants or of the wild-type parent strain.

Reversion experiments with EMS were undertaken to characterize the combined loss of nuclease, coagulase, and β-hemolysin activities. Four independent revertants were obtained from a class II mutant and six were obtained from a class III mutant, at a frequency of 0.2 to 0.3%. Two were isolated on DNA-AO-agar and eight were isolated on sheep blood-agar. Upon plating on the other test media or after growth in liquid culture, all revertants regained coagulase, deoxyribonuclease, and β-hemolysin activities. The class III revertants were still untypeable by standard phages, and they remained less susceptible than wild-type cultures to lysostaphin. It must be
Table 1. Nuclease-deficient mutants of Staphylococcus aureus Foggi strain

<table>
<thead>
<tr>
<th>Class of strain</th>
<th>Nuclease</th>
<th>CRM</th>
<th>Coagulase</th>
<th>β-hemolysis</th>
<th>α-hemolysis</th>
<th>Lysostaphin susceptibility</th>
<th>Phage type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Normal</td>
<td>53/83</td>
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<tr>
<td>I</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Normal</td>
<td>53/83</td>
</tr>
<tr>
<td>II</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Normal</td>
<td>53/83</td>
</tr>
<tr>
<td>Reversion with EMS</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Normal</td>
<td>53/83</td>
</tr>
<tr>
<td>III</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Resistant</td>
<td>Untypable</td>
</tr>
<tr>
<td>Reversion with EMS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Resistant</td>
<td>Untypable</td>
</tr>
</tbody>
</table>

* Abbreviations: CRM, cross-reacting material; EMS, ethyl methane sulfonate.

concluded that the change in phage type and lysostaphin susceptibility represented a second, unrelated mutation.

Addition of DNA (about 100 μg/ml) or of thymidine or thymine (1 to 10 mM) to the culture medium did not alter the nuclease activity in the supernatant fluid. Thus, neither induction nor end-product inhibition could be demonstrated in preliminary studies of the regulation of production of this extracellular enzyme.

DISCUSSION

The phenotypic patterns of mutation for nuclease-deficient strains are given in Table 1. The single mutant with isolated nuclease deficiency (class I) lacked antigenically cross-reacting material and was of no value for structure-function studies of substitutions in the nuclease sequence. The site of mutation is uncertain, but termination, frameshift, or deletion of the nuclease structural gene, as well as alteration in a hypothetical regulatory gene, are all possibilities. Mutations resulting in termination codons or frameshift in the nuclease structural gene would lead to products that would not cross-react, since only nuclease polypeptide chains with intact C-terminal portions will precipitate with antinuclease antibody. Our immunological studies have shown that a nuclease sequence fragment containing amino acid residues 1 to 126 does not precipitate with antibody (20) and that the carboxy-terminal fragment (residues 127 to 149) contains one of the two major antigenic determinants in the sequence (G. S. Ommen, D. A. Ontjes, and C. B. Anfinsen, 1970, Biochemistry 9: 313–321).

The lack of CRM+ missense mutants of nuclease may be due to the rejection in the screening technique of "leaky mutants," in which the altered nuclease molecule or possibly another, minor enzyme with deoxyribonuclease activity (22) could have been functional.

The class II and class III mutants exhibited a combined loss of nuclease, coagulase, and β-hemolysin. The recovery of all three functions in revertants is suggestive of a coordinate control mechanism for these extracellular enzymes. Loss of a hypothetical plasmid carrying genes for these enzymes and the possibility of deletion of these genes from a chromosomal location are ruled out by the isolation of revertants. In studies that may provide data complementary to these findings in the nuclease-deficient mutants, McClatchy and Rosenblum (14) reported that 11 of 20 α-hemolysin mutants also lacked fibrinolysin and that both traits reverted to normal in recombination experiments (15). In studies of penicillinase production, Harmon and Baldwin (10) noted a mutant which failed to produce detectable amounts of α-hemolysin, fibrinolysin, lipase, gelatinase, and caseinase, but which had normal deoxyribonuclease, coagulase, and β-hemolysin activities. Reversion was not attempted. These few experimental findings may indicate that two (or more) sets of genes for extracellular enzymes are under coordinate, but separate, control. Mapping and linkage data in S. aureus are very limited, except for the markers associated with the penicillinase plasmid (18) and the recent reports of possible operons for protoheme (21) and lysine (3) biosynthesis.

Very little is known about the regulation of production or the process of release of these extracellular enzymes. No nuclease could be detected intracellularly in the wild type, and there was no evidence that nuclease was retained intracellularly in any of the nuc− mutants. Although induction and product inhibition have not been demonstrated, it is possible that regulation is subject to coordinate induction, so that an inducer of one enzyme leads to production of several.

The nuclease-deficient mutants reported here point to patterns of coordinate control of synthesis and secretion of the enzymes so characteristic of pathogenic strains of S. aureus. Such nuc− strains may permit transformation experiments and may be useful for studies of the role of the extracellular enzymes in pathogenicity.

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LITERATURE CITED


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