Genetics of Serum Resistance in *Neisseria gonorrhoeae*: The *sac-1* Genetic Locus

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A genetic locus affecting susceptibility to the bactericidal activity of normal human serum has been designated *sac-1*. This locus was shown to be closely linked to, but not identical with, a second locus (designated *nmp-2*) that affects protein 1 of the outer membrane. The *sac-1* locus could be linked to known antibiotic resistance markers on the gonococcal chromosome by genetic transformation.

A correlation between virulence and resistance to the bactericidal activity of human serum has been observed for several different gram-negative organisms, including *Escherichia coli* (20) and *Pseudomonas aeruginosa* (23). Strains isolated from the blood are usually serum resistant, whereas isolates from stool, urine, or mucosal surfaces are often sensitive to killing by serum. This relationship also has been shown to exist for infections caused by *Neisseria gonorrhoeae*. Strains that cause disseminated gonococcal infection are usually penicillin sensitive (22), exhibit unusual nutritional requirements (9), and are serum resistant (14). In contrast, gonococcal strains that produce local inflammation are frequently serum sensitive (1). In an epidemiological study, Eisenstein et al. (3) showed that the ability to cause disseminated disease correlates with two independent properties: penicillin sensitivity and serum resistance. These observations suggest that the property of serum resistance may be an important factor in the ability of the gonococcus to cause bacteremic disease.

Studies on the genetic basis of the serum resistance of the gonococcus should be useful in determining the relationship between serum resistance and other properties of the cell and in identifying the components of the cell surface involved in resistance to the bactericidal action of serum. A limited number of such studies have been done. Mayer et al. (12) reported that genetic lesions responsible for auxotrophy of strains causing disseminated gonococcal infection are unlinked to a genetic locus for serum resistance. Spratt et al. (18) described a genetic locus (*sac-2*) that affects the level of serum resistance. The biochemical basis for differences in resistance due to this locus was not studied. Hildebrandt et al. (6) reported that transforma-

tion of a serum-sensitive gonococcal strain to serum resistance was accompanied by simultaneous structural and antigenic changes in protein 1 of the outer membrane (the principal outer membrane protein). These authors suggested that serum resistance of gonococci is related to the possession of a protein 1 of characteristic subunit molecular weight and antigenic structure.

In this paper, we report the identification of a genetic locus affecting the level of serum resistance of the gonococcus. This locus, designated *sac-1*, was closely linked to, but not identical with, a locus that affected protein 1.

MATERIALS AND METHODS

Strains used. The strains of *N. gonorrhoeae* used in these studies are listed in Table 1. Growth conditions and media have previously been described (13). Determination of colony opacity or transparency was made according to the procedures of Swanson (19).

Genetic transformation. Procedures for preparation of transforming deoxyribonucleic acid (DNA) and genetic transformation were as previously described (13). Selection of antibiotic-resistant transformants was made on agar plates of GCBA-DS medium (13) containing the following concentrations of antibiotics: rifampin (Rif), 2 µg/ml; streptomycin (Str), 100 µg/ml; spectinomycin (Spc), 200 µg/ml. For selection of serum-resistant transformants, serum-sensitive competent cells were mixed with saturating amounts of transforming DNA from a serum-resistant strain. After incubation at 37°C with shaking for 4 to 5 h, deoxyribonuclease (Worthington) was added to a final concentration of 50 µg/ml, and the mixture was incubated for an additional 5 min. The transformation mixture was mixed with pooled normal human serum (NHS), to a final serum dilution of 1:4, and incubated for 30 to 45 min at 37°C with shaking. Survivors of this treatment were tested for level of serum resistance in the standard serum bactericidal assay.

Serum bactericidal assay. The time course of

TABLE 1. Strains of *N. gonorrhoeae* used

Strain	Relevant genotype	Serum susceptibility ^a	Apparent mol wt of protein 1 ^b (10 ³)	Source
FA19	<i>sac-1</i> ^c	R	36.5	A. Reyn
F62	<i>sac-1</i> ⁺	S	37.2	D. Kellogg
FA600	<i>sac-1</i>	R	36.5	Serum-resistant transformant: FA19 × F62
FA671	<i>sac-1</i> ^c	R	36.5	Second-step serum-resistant transformant: FA19 × FA600
FA114	<i>rif-1 spc-3 sac-1</i> ^{c,d}	R	36.5	Rif ^r Spc ^r derivative of strain FA19 (13)
FA101	<i>str-7 sac-1</i> ^c	R	36.5	Str ^r transformant: FA47 (17) × FA19
FA610	<i>str-7 sac-1</i> ⁺	S	37.2	Str ^r transformant: FA101 × F62
FA630	<i>str-7⁺ sac-1</i> ⁺	S	37.2	Str ^r serum-sensitive derivative of FA19: FA610 × FA19
FA635	<i>spc-3 sac-1</i> ⁺	S	36.5	Spc ^r transformant: FA130 (Spc ^r derivative of FA19; reference 13) × F62
FA140	<i>sac-1</i> ^c	R	39.0	(17)
FA198	<i>spc-3 sac-1</i> ^c	R	39.0	Spc ^r derivative of FA140 (17)
FA488	<i>sac-1</i> ⁺	S	37.2	<i>mtr penA</i> ^r derivative of strain F62: FA48 (17) × F62
FA640	<i>spc-3 sac-1</i>	R	39.0	Spc ^r transformant: FA198 × FA488

^a Serum-resistant strains are designated R; serum-sensitive strains are designated S.

^b Apparent molecular weight of protein 1 was determined by SDS-PAGE.

^c These strains contain at least one other genetic marker affecting the level of serum resistance besides the *sac-1* marker.

^d *rif-1* is a locus for rifampin resistance; *spc-3* is a locus for spectinomycin resistance (13).

^e *str-7* is a locus for streptomycin resistance (13).

^f *mtr* and *penA* are loci for low-level penicillin resistance (17).

complement-dependent killing of gonococci by NHS was measured by the method previously described by Lee et al. (11). In genetic experiments, serum resistance was usually scored by exposing gonococci to different dilutions of NHS for 30 min at 37°C in wells of microtiter trays, as described by Eisenstein et al. (3). Results were expressed as percent survival (in colony-forming units), compared with controls. A culture was scored as resistant to a given dilution of serum if survival was ≥70%. Strains classified as serum resistant were generally resistant to a 1:4 dilution of normal serum; strains classified as serum sensitive were sensitive to at least a 1:16 dilution of serum. NHS pools were prepared from blood collected from six to ten laboratory workers who had not been previously infected with *N. gonorrhoeae*.

Isolation of outer membranes. Procedures for cell lysis in a French pressure cell, isolation of a crude membrane fraction, and purification of outer membranes by Sarkosyl (Geigy Chemicals) extraction have been described previously (5).

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done by the method of Shapiro et al. (15), as previously described (2). The apparent molecular weight of proteins was determined by SDS-PAGE, using reference proteins of known molecular weight. A protein with an apparent molecular weight of 36,500 is referred to as 36.5K.

RESULTS

Strain FA19 is fully serum resistant, with a protein 1 of apparent molecular weight of 36.5K. Strain F62 is highly sensitive to killing by NHS and has a protein 1 of 37.2K. Figure 1 illustrates

the difference in serum sensitivity of these strains. The serum resistance property of strain FA19 was transferred into strain F62 by genetic transformation, with selection made for resistance to a 1:4 dilution of NHS. The resulting transformants were more resistant to serum than the recipient strain was, but were less resistant than the donor strain (Fig. 1). A second genetic transformation, with strain FA19 as the donor and strain FA600, one of the intermediate-level serum-resistant transformants, as the recipient, yielded transformants that were identical to strain FA19 in serum susceptibility (Fig. 1).

It has been reported that transparent and opaque colony variants of a single gonococcal strain can have different levels of susceptibility to killing by serum (10). The differences in serum sensitivity of strains FA19, F62, and FA600 were not due to variations in colony opacity, since transparent and opaque variants of each of these strains showed similar susceptibility to serum killing.

The proteins of the outer membrane of several of the intermediate-level serum-resistant transformants were analyzed by SDS-PAGE (Fig. 2). Each had acquired the donor protein 1 of 36.5K, which suggested that protein 1 might play a direct role in serum resistance.

In further genetic experiments, however, the difference in serum resistance between strains FA19 and F62 was genetically separated from the difference in protein 1 of the two strains.

FIG. 1. Serum antibody-complement-mediated killing of strains of *N. gonorrhoeae*. Cells were incubated with a 1:4 dilution of NHS at 37°C, and extent of survival was determined as described in the text. Symbols: ●, strain FA19 (serum resistant, protein 1_{36.5K}); ■, strain F62 (serum sensitive, protein 1_{37.2K}); ○, strain FA600 (intermediate-level serum-resistant transformant, protein 1_{36.5K}); □, strain FA671 (fully serum-resistant transformant, protein 1_{36.5K}); △, strain FA635 (serum-sensitive transformant, protein 1_{36.5K}).

Preliminary experiments showed that the difference in serum resistance of the two strains could be genetically linked to several antibiotic resistance markers that are located in a cluster on the gonococcal chromosome (4, 16). A genetic transformation was done, with strain FA630 (*rif-I*⁺ *str-7* *spc-3*⁺, serum sensitive, protein 1_{37.2K}) as the donor. The recipient was strain FA114 (*rif-I* *str-7*⁺ *spc-3*, serum resistant, protein 1_{36.5K}). Streptomycin-resistant transformants were selected and scored for level of serum resistance. Transformants were also scored for apparent molecular weight of protein 1 by SDS-PAGE of lysates of whole cells. A total of 96 streptomycin-resistant transformants were classified into four groups: three were serum sensitive, with protein 1 of 37.2K; four were serum sensitive, with protein 1 of 36.5K; four were serum resistant, with protein 1 of 37.2K; and 85 were serum resistant, with protein 1 of 36.5K. All four possible combinations of serum resistance and protein 1 apparent molecular weight were recovered, with 8 of the 96 transformants showing recombination between serum resistance phenotype and protein 1 phenotype.

Separation of serum resistance and protein 1 phenotype of strains FA19 and F62 also occurred when a spectinomycin-resistant (*spc-3*) derivative of strain FA19 was crossed by transformation with strain F62 (data not shown). Spectinomycin-resistant, serum-sensitive transform-

ants that acquired the 36.5K protein 1 of the donor strain were indistinguishable in susceptibility to serum killing from the serum-sensitive recipient strain F62, which had the 37.2K protein 1 (Fig. 1). Therefore, the difference in level of serum resistance of strains FA19 and F62 could not be due to the difference in protein 1 between them. We believe that there is a genetic locus

FIG. 2. SDS-PAGE of outer membrane proteins of serum-sensitive and serum-resistant strains. A, strain FA140 (serum resistant, protein 1_{39K}); B, strain FA488 (serum sensitive, protein 1_{37.2K}); C, strain FA640 (serum-resistant transformant, protein 1_{39K}); D, molecular weight standards [bovine serum albumin (67,000), ovalbumin (45,000), myoglobin (17,800), cytochrome c (12,400)]; E, strain FA19 (serum resistant, protein 1_{36.5K}); F, strain F62 (serum sensitive, protein 1_{37.2K}); G, strain FA600 (serum-resistant transformant, protein 1_{36.5K}).

that affects the level of serum resistance, which is located near the *str-7* locus on the gonococcal chromosome, and which is independent of the protein 1 phenotypes found in strains FA19 and F62. We have designated this locus as *sac-1* (serum antibody-complement) and have arbitrarily designated the allele for serum sensitivity in strain F62 as *sac-1*⁺ and that for serum resistance in strain FA19 as *sac-1*. The *sac-1* locus was closely linked to the genetic locus responsible for the change in apparent molecular weight of protein 1 from 36.5 to 37.2K, which we designated *nmp-2* (new membrane protein).

In similar experiments, a serum-resistant strain with a protein 1 of 39K (strain FA198) was used as the donor of the *sac-1* marker to strain F62. Most of the transformants that acquired the donor serum resistance also showed an increase in apparent molecular weight of protein 1 to 39K (Fig. 2). It was possible, however, to recover serum-sensitive transformants that acquired the 39K protein 1 of strain FA198 (data not shown). Therefore, the *sac-1* marker was also independent of the 39K protein 1 of strain FA198.

The position of the *sac-1* locus on the gonococcal chromosome was determined by genetic mapping experiments in which the *rif-1*, *str-7*, and *spc-3* antibiotic resistance loci were used as reference markers. These loci are well characterized, and their relative positions on the gonococcal chromosome have been previously established (13). Reciprocal transformation crosses were carried out between strains FA630 (*rif-1*⁺ *str-7*⁺ *spc-3*⁺ *sac-1*⁺) and FA114 (*rif-1*⁺ *str-7*⁺ *spc-3* *sac-1*). The results of these experiments are shown in Table 2 and suggest a gene order of *rif-1*—*str-7*—*spc-3*—*sac-1*. When *rif-1* was the selected marker, there were three transformants that were Rif^r Str^r Spc^s serum^r, which were presumably generated by double crossover events. Although this number is somewhat higher than might be expected, the frequency of

occurrence of the other recombinant classes was consistent only with the suggested order of these genetic markers. The genetic map constructed from these data is shown in Fig. 3. The positions of the *nmp-1* and *penB* genetic loci, which are included on the genetic map, were determined previously (2). Due to the close linkage between the *sac-1*, *nmp-2*, *nmp-1*, and *penB* loci, we have been unable to determine their relative positions, either in these experiments or in additional mapping experiments (data not shown).

DISCUSSION

Our results show that the genetic locus we have designated *sac-1* does have an effect on the level of serum resistance of the gonococcus. These results also illustrate the complex nature of the mechanisms that can give rise to differences in susceptibility to serum killing. Transformation of a serum-susceptible strain to the level of resistance of donor strain FA19 required two separate transformation events. Strains FA19 and F62 must differ in at least two genetic

FIG. 3. Genetic map derived from the data in Table 2. The numbers represent the frequency of recombination between two genetic markers. Arrows point from the selected donor marker to the unselected, scored marker. Genetic markers in parentheses could not be ordered relative to each other. Map distances are not drawn to scale. *nmp-1* is a genetic locus affecting protein 1 of the outer membrane; *penB* is a locus for relatively nonspecific, low-level antibiotic resistance (2, 17). The *sac-1* (serum resistance) and *nmp-2* (altered protein 1) loci are described in the text.

TABLE 2. Mapping order from reciprocal transformation test crosses^a

Cross	Selected marker 1000	Unselected donor markers			Total colonies tested	No. in recombinant class:							
		0100	0010	0001		1000	1001	1010	1011	1100	1101	1110	1111
Recipient strain (0000): <i>rif-1</i> ⁺ <i>str-7</i> ⁺ <i>spc-3</i> ⁺ <i>sac-1</i> ⁺ (FA630); donor strain DNA (1111): <i>rif-1</i> ⁺ <i>str-7</i> ⁺ <i>spc-3</i> <i>sac-1</i> (FA114)	<i>rif-1</i>	<i>str-7</i> ⁺	<i>spc-3</i>	<i>sac-1</i>	96	63	3	0	0	28	0	1	1
	<i>spc-3</i>	<i>rif-1</i>	<i>str-7</i> ⁺	<i>sac-1</i>	100	43	12	34	11	0	0	0	0
Recipient strain (0000): <i>rif-1</i> <i>str-7</i> ⁺ <i>spc-3</i> <i>sac-1</i> (FA114); donor strain DNA (1111): <i>rif-1</i> <i>str-7</i> ⁺ <i>spc-3</i> ⁺ <i>sac-1</i> ⁺ (FA630)	<i>str-7</i>	<i>rif-1</i> ⁺	<i>spc-3</i> ⁺	<i>sac-1</i> ⁺	96	23	0	20	2	39	1	7	4

^a Suggested gene order: *rif-1*—*str-7*—*spc-3*—*sac-1*. "1" and "0" refer to genetic markers of the donor and recipient strains, respectively.

loci that can affect the level of serum resistance: *sac-1* and another, as yet unidentified, genetic locus. This conclusion is compatible with the work of Hildebrandt et al., who also found that transformation of a serum-sensitive strain to full serum resistance required two transformation events (6).

Genetic analysis proved to be useful in studying the possible association of protein 1 phenotype with differences in serum resistance due to *sac-1*. In our initial transformation experiments, when serum-resistant transformants were selected, it appeared that protein 1 might have a role in serum resistance. When selection was made for a linked antibiotic resistance marker, however, it was possible to separate protein 1 phenotype from the serum resistance phenotype of the transformants. This was shown with the 36.5, 37.2, and 39K forms of protein 1. The apparent association of these properties was due to the fact that the genetic loci affecting serum resistance and protein 1 phenotype were located close together on the gonococcal chromosome. We do not know what mechanism is involved in the effect of the *nmp-2* locus on protein 1, but our results showed that the action of the *sac-1* locus was not dependent on the presence of a particular allele of *nmp-2*. This result is not necessarily inconsistent with that of Hildebrandt et al. (6), who concluded that a particular form of protein 1 might be important in serum resistance. The apparent molecular weight and antigenicity of protein 1 vary among gonococcal strains (7). We have identified *sac-1* as a locus that is independent of the 36.5, 37.2, and 39K forms of protein 1 used in these experiments. However, there may be other protein 1 types that do have an effect on the level of serum resistance, possibly by altering the exposure of another target antigen of bactericidal activity.

Although our results indicate that the *sac-1* locus probably affects some component of the cell other than protein 1, we do not know the identity of the component. There have been several reports that gonococcal lipopolysaccharide is a target antigen for antibody-complement killing (8, 21), which suggested to us that lipopolysaccharide might be a candidate for the component affected by *sac-1*. In a preliminary communication (4), we reported that lipopolysaccharide isolated from strains F62 and FA600 differed in its ability to inhibit the bactericidal action of NHS. However, in further experiments with additional preparations of lipopolysaccharide from these two strains, as well as with another transformant similar to FA600, we have found the magnitude of the difference and the amount of lipopolysaccharide required to show

that difference are variable. Also, we have been unable to detect any structural difference in the lipopolysaccharide from these serum-resistant and serum-sensitive strains by SDS-PAGE, gas-liquid chromatography (data not shown), or immunological methods (M. Apicella, personal communication). These results compel us to be cautious in implicating lipopolysaccharide as the component that is altered in strains differing at the *sac-1* genetic locus. Further studies will be needed to resolve this question.

Genetic mapping experiments showed that the *sac-1* locus could be genetically linked to, and ordered relative to, several well-characterized antibiotic resistance loci. The order of the *rif-1*, *str-7*, and *spc-3* genetic loci determined in these experiments is consistent with previously published results (13). Spratt et al. (18) have recently described a genetic locus, designated *sac-2*, that also maps near this region of the gonococcal chromosome. These authors believe that *sac-2* is located farther away from the *spc* locus than an *arg* marker that shows 1.3% cotransformation with *spc*. In contrast, our results indicated that the cotransformation frequency between *sac-1* and *spc-3* was 23%. These differences in linkage values obtained in their experiments and in our own suggest that *sac-1* and *sac-2* are different genetic loci.

It is clear that there are multiple genetic loci that can have an effect on the level of serum resistance of the gonococcus. Further studies will be needed to identify the cellular components affected by these loci and to assess their role in the ability of the gonococcus to establish bacteremic disease.

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