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Mutational Loss of Sensitivity to Mutacin GS-5 in *Streptococcus pyogenes*: Characterization of a Mutant Deficient in Receptor Protein

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By means of a stepwise selection procedure, mutants capable of growing in the presence of relatively high multiplicities of a bacteriocin from *Streptococcus mutans* GS-5 were obtained from a sensitive strain of *Streptococcus pyogenes*. Mutacin-neutralizing activity of cell extracts containing receptor protein was examined in one variant that adsorbed ⅙ the amount of bacteriocin adsorbed by the parent strain under conditions equivalent to "saturation." Partially purified receptor protein from both parent and mutant cells neutralized an equivalent amount of bacteriocin on a weight-to-weight basis, indicating that in vitro there was no significant difference in affinity for the mutacin between the respective receptor fractions. Cell extracts from the mutant, solubilized by treatment with trichloroacetic acid, neither neutralized mutacin activity nor interfered with receptor protein-mediated mutacin neutralization in vitro. The mutant phenotype may thus represent a cell surface density of receptor protein which results in the adsorption of sublethal amounts of mutacin. The mutant retained its sensitivity to other mutacins, e.g., those produced by strains LM-7 and BHT of *S. mutans*, and did not differ from wild-type cells with respect to either detergent sensitivity (sodium lauryl sulfate and Triton X-100) or to inhibition by penicillin, rifampin, bacitracin, erythromycin, and tetracycline.

Several strains of *Streptococcus mutans* produce bacteriocin-like substances which have been designated as mutacins (7). The mutacin elaborated by a serotype *c* strain, GS-5, is a 20,000-dalton protein which is bactericidal for streptococci representative of Lancefield groups A, C, D, G, L, and O (13). Exocellular synthesis of this mutacin is noninducible (13), occurs at the end of exponential growth (13), and in liquid media may be enhanced by the presence of unidentified thermolabile and dialyzable substances (4).

A surface glycoprotein, which may be analogous to bacteriocin receptor substances found in other organisms (1, 3, 10, 12), has been isolated from a sensitive strain of *Streptococcus pyogenes* (14). Although it has been shown that other surface polymers such as the group and type antigens do not affect adsorption of this mutacin (13), nothing is known about the initial steps in mutacin-cell interaction or the causal basis for cell death. The identity of a "target" entity in sensitive cells remains obscure.

One approach to resolving some of these questions is to examine the range of phenotypic modifications that alter the susceptibility of sensitive cells. To this end, I have isolated a number of mutants derived from a sensitive strain of *S. pyogenes*. In this paper some properties of one of these variants are described.

MATERIALS AND METHODS

Organisms. The bacteriocin-sensitive parent strain (MJP-2) is a beta-hemolytic group A clinical isolate of *S. pyogenes* obtained from M. Pickett, Department of Microbiology, University of California, Los Angeles. Strain GS-5 *S. mutans*, belonging to Bratthall serogroup *c* (2), was obtained from M. Newman, Section of Periodontology, University of California-Los Angeles School of Dentistry.

Media. APT broth was purchased from BBL Microbiology Systems (Cockeysville, Md.) and, for use in pour plates, was solidified with 1% agar. APT-YE medium is one-half strength APT plus 4% yeast extract as devised by DeLisle (4) and was used exclusively for mutacin production after filtration (0.45-μm pore size; Nalgene Corp.).

Mutacin purification. One-liter cultures of *S. mutans* GS-5 in APT-YE, incubated overnight at 37°C, were used to obtain purified concentrates of the mutacin. After removal of the cells by centrifugation (12,000 × *g*, 30 min), the supernatant was adjusted to pH 7.0, and solid $(NH_4)_2SO_4$ was slowly added to 80% saturation. The precipitate formed after 16 to 20 h at 4°C was then pelleted by centrifugation (27,000 × *g*, 20 min) and dissolved in 20 ml of 0.05 M phosphate buffer (pH 6.2). The protein solution was dialyzed against 1,000 volumes of the same buffer and then

applied to a Cellex D (BioRad Labs) column (2.5 by 25 cm) equilibrated in 0.05 M buffer. After a wash with 0.5 M buffer, 2.25-ml fractions were eluted from the column with a linear 0.05 to 0.5 M phosphate gradient and assayed for inhibitory activity as described below. The mutacin-containing fractions (eluting at the 0.12 to 0.2 M region of the gradient) were pooled, concentrated by pervaporation in 0.25-in (ca 0.64-cm) dialysis tubing to 5 ml, and then applied to a BioGel P-100 column (2.5 by 100 cm) equilibrated in 0.05 M buffer. Fractions of 4.5 ml were collected at a flow rate of 20 ml/h and assayed for activity. The mutacin-containing portion of the eluate was then pervaporated as described above to a protein concentration of 2 mg/ml and stored at −20°C.

Quantitation of the mutacin. Mutacin titers were determined by incubating 5×10^7 colony-forming units of washed mid-exponential-phase indicator cells in 1 ml of 0.2 M phosphate buffer with 10-μl samples of column-purified concentrate for 30 min at 37°C. The viable count was then assayed after 24 h of incubation of APT-agar pour plates inoculated with appropriately diluted samples. Specific activity in this study is expressed as killing units (KU) per microgram of protein, with 1 KU equivalent to the multiplicity of mutacin estimated to kill a single cell, assuming that an average of four cells constitute a colony-forming unit. The yield of mutacin in the six preparations used ranged from 1.6×10^6 to 2.0×10^6 KU/μg of protein for column-purified concentrates.

Protein determination. The BioRad reagent (BioRad Labs) was used with lysozyme as a standard.

Isolation of mutants. Because the loss of sensitivity to mutacin GS-5 appears to develop in a stepwise manner in this strain of *S. pyogenes*, a culture of parent cells derived from a single colony was appropriately diluted and then mixed with 10-ml volumes of APT-agar at 50°C. Mutacin was then added at multiplicities ranging from 1 to 5 KU per cell. Pour plates prepared from such mixtures were then examined after 48 h of incubation at 23°C. This temperature was chosen to avoid selecting against any temperature-sensitive mutants that might occur. Colonies growing in plates containing the highest mutacin concentration were picked, purified by two rounds of single-colony isolation, and then grown overnight in 3 ml of APT broth containing mutacin at the multiplicity at which they were originally selected. The pour-plate procedure was then repeated for isolating survivors at greater multiplicities until a KU/cell ratio of 80 was reached. No mutagens were used in this study.

Receptor protein preparations. Sonicated cell extracts were obtained from 2-liter cultures by essentially the same method as that described by Perry and Slade (14). After incubation for 16 h at 37°C, cells were harvested by centrifugation, washed three times with 0.05 M phosphate buffer, and sonicated for 1 min on ice in 15 ml of 0.02 M buffer (Branson W200P Sonifier, microtip, setting no. 6), and the protein in the supernatant that was obtained after 30 min of centrifugation at 27,000 × *g* was concentrated by precipitation with $(NH_4)_2SO_4$ at 80% saturation. The precipitates were dissolved in 0.2 M buffer at protein concentrations of 25 mg/ml after pelleting by centrifugation (27,000 × *g*, 30 min). Preparations were stored at −20°C either as a crude extract or after being resolved in fractions of greater specific activity by sieving 0.7-ml volumes through a BioGel A 1.5 column (1.5 by 50 cm).

Trichloroacetic acid extracts. Washed cells from 2-liter overnight cultures were suspended in 20 ml of cold 10% (wt/vol) trichloracetic acid and extracted by slow stirring for 16 h at 4°C. The supernatant obtained after centrifugation (10,000 × *g*, 30 min) was then mixed with 5 volumes of ethanol and held at 4°C for 16 h. The precipitate formed was concentrated by centrifugation (10,000 × *g*, 30 min) and dissolved in 5 ml of cold 1 trichloroacetic acid, and then 2 volumes of ethanol was added to selectively precipitate teichoic acids in the preparation. After 16 h at 4°C, the precipitate was concentrated as described and dissolved in 2 ml of water, and its carbohydrate content was assayed by the method of Dubois et al. (5). Protein was also measured as already described.

Mutacin-neutralization assays. For whole cells, tests for mutacin-binding activity were conducted by incubating 8 g (1.5×10^7 KU) of the mutacin with or without (control) washed cells in 0.02 M phosphate buffer for 30 min at 37°C in an incubation volume of 1 ml. Cell-free supernatants obtained after 10 min of centrifugation at 10,000 × *g* were then incubated with 1 ml containing 10^7 colony-forming units of mid-exponential-phase cells for 30 min, and the decrease in killing activity was determined by plating appropriate dilutions. The mutacin-neutralizing activity of cell-free extracts was assayed in a similar way except that the volume of reaction mixtures was 0.5 ml and they were not centrifuged before addition of indicator cells for the determination of residual activity.

RESULTS

Isolation of mutants. The survival curves shown in Fig. 1 depict the protocol used for the selection and isolation of mutant S4780, the properties of which are presented herein. The data suggest that survival in the presence of increasing amounts of mutacin GS-5 requires several mutational steps, each of which results in a relatively small loss of susceptibility. Furthermore, a sizeable proportion (80 to 85%) of the colonies isolated from plates containing mutacin at less than 10 KU per cell did not grow when transferred to broth containing the same concentration of bacteriocin. This finding is similar to that observed with colonies growing within inhibition zones formed by stab cultures of *S. mutans* GS-5 when the classic agar overlay technique (6) was used. The differential susceptibility of agar- and broth-grown cells to bacteriocins has been noted in other systems (8) and may also obtain for *S. pyogenes* and this mutacin.

Growth of mutant S4780 in the presence of mutacin GS-5. S4780 is one of eight isolates obtained that were able to grow in the presence of mutacin GS-5 at a multiplicity of 80 KU in liquid media at 23°C as well as 37°C. As shown

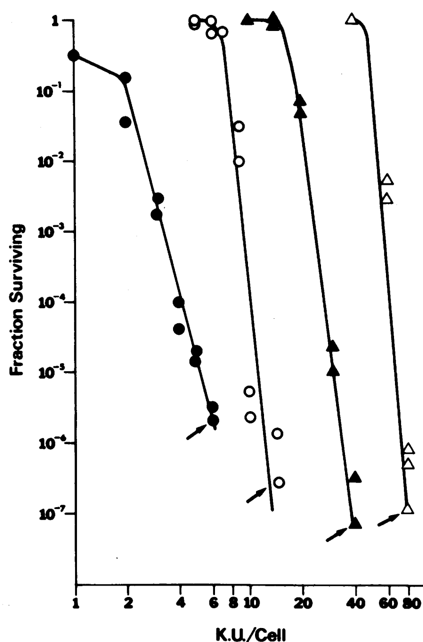

FIG. 1. *Survival curves of parent S. pyogenes and mutants derived from parent clone. The arrows indicate points at which populations of colonies were picked for isolation of the next step mutant. S4780 was a colony found on plates containing GS-5 mutacin at multiplicity of 80 KU per cell.*

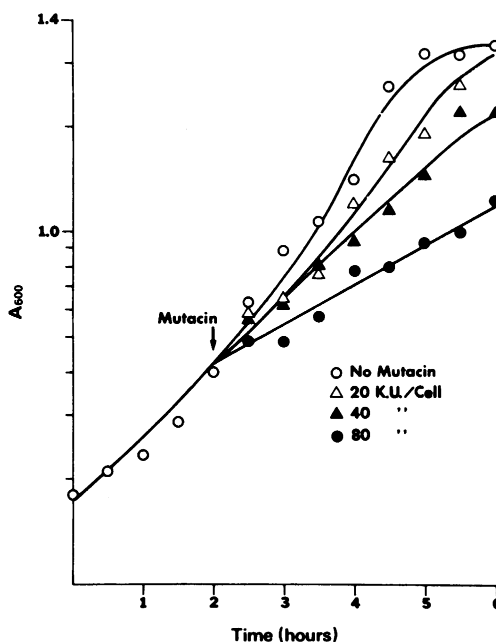

FIG. 2. *Growth of mutant S4780 at different multiplicities of GS-5 mutacin. An overnight culture of S4780 was diluted 1:10 in 100 ml of APT prewarmed at 37°C; after 2 h, the culture was divided into 25-ml subcultures containing no mutacin (control) and mutacin at indicated multiplicities. Cell mass was monitored by measuring the adsorbance of samples at 600 nm (A_{600}).*

in Fig. 2, its mass doubling time increased when the multiplicity of mutacin GS-5 was increased, indicating that the bacteriocin does mediate detectable effects on the growth of this variant. When compared to parent cells (data not presented), S4780 exhibited no significant differences in susceptibility to either of the detergents sodium lauryl sulfate or Triton X-100 or to penicillin, rifampin, bacteriocin, erythromycin, and tetracycline. S4780, however, remained fully sensitive to inhibition by other mutacinogenic strains of *S. mutans* in overlay cultures, e.g., LM7 (serotype *e*) and BHT (serotype *b*).

Mutacin adsorption by whole cells. By measuring the residual activity of mutacin preparations after preincubation with thoroughly washed cells and under ionic conditions that appeared to minimize nonspecific binding (0.2 M buffer), it was found that S4780 cells adsorb about ⅙ the amount of bacteriocin bound or neutralized by wild-type cells (Fig. 3). These assays also indicate that 1.5×10^7 KU of mutacin can be neutralized by 8×10^8 colony-forming units or 3.2×10^9 single cells. If it is assumed that, since the mutacin preparations used represent a 300-fold increment in purification, 1.5 $\times 10^7$ KU represents about 4×10^{13} molecules of mutacin GS-5, then a single wild-type cell can

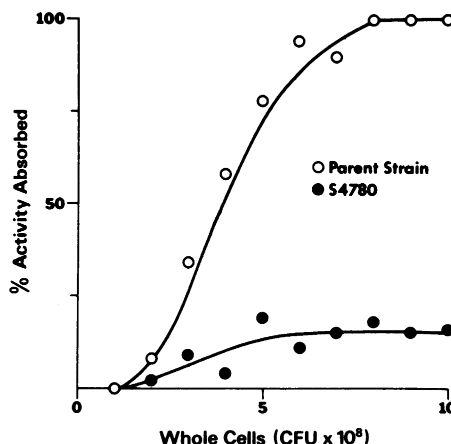

FIG. 3. *Mutacin-neutralizing activity of whole-cell suspensions. Diluted portions (1 ml) of cells washed three times with 0.2 M phosphate buffer (pH 6.2) were incubated for 30 min at 37°C with 1.5×10^7 KU of mutacin. Residual activity was determined after centrifugation of cells (10,000 × g, 10 min) by incubating 10^7 mid-exponential-phase wild-type cells with the supernatants and then assaying for the decrease in killing activity by plating appropriately diluted samples. Each point is the average of three determinations.*

adsorb as much as 10^4 molecules of mutacin, and mutant S4780 cells adsorb almost an order of magnitude less. These estimates, however, may have limited applicability because during the chainlike growth of streptococcal cells, portions of the cell surface, e.g., areas of intercellular adhesions, may not be exposed to the extracellular milieu and consequently are unavailable for mutacin binding.

Neutralization of mutacin by crude sonic extracts. When sonic extracts prepared from parent and mutant cells were assayed for mutacin-neutralizing activity, inactivation curves exemplified by the data in Fig. 4 were obtained with four independent sets of extracts. When assayed for proteolytic activity by methods described by others (14), both wild-type and mutant extracts were negative.

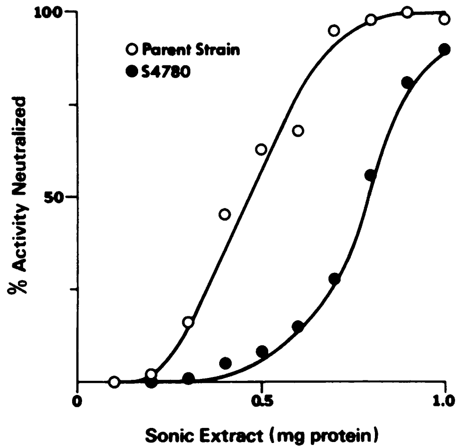

FIG. 4. *Mutacin-neutralizing activity of extracts obtained by sonicating whole cells. Overnight 2-liter cultures were used to obtain protein concentrate (25 mg/ml) as described in the text. Extract at indicated concentrations of protein was incubated with 1.5 × 10^7 KU of mutacin at 37°C for 30 min in a reaction volume of 0.5 ml. Residual activity was assayed as described in the legend to Fig. 3.*

Neutralization of mutacin with column-purified extracts. To determine whether the disparity described above was due to some intrinsic difference in the affinities of wild-type and mutant receptor protein for the mutacin, the sonic extracts were fractionated by sieve chromatography as depicted by the elution profiles shown in Fig. 5. Mutacin-neutralizing activity was found at identical positions (equivalent to an M_r of 9 × 10^4 to 10 × 10^4 as suggested by precalibration of the column with molecular weight standards), and the pooled fractions from each represented a sixfold increment in specific activity, although the total amount of receptor recovered from the mutant extract was less than that for the wild type. When these fractions were compared for neutralizing activity, no significant differences were observed (Fig. 6). This result suggested that receptor substance from mutant cells at this level of purification can function in the same manner as wild-type protein. The mutant phenotype may therefore represent a simple depletion of receptor substance on the cell surface.

Effects of trichloroacetic acid extracts on mutacin activity. Cell extracts obtained by ethanol precipitation of trichloroacetic acid-soluble surface material, from either the parent strain or S4780 cells, did not neutralize GS-5

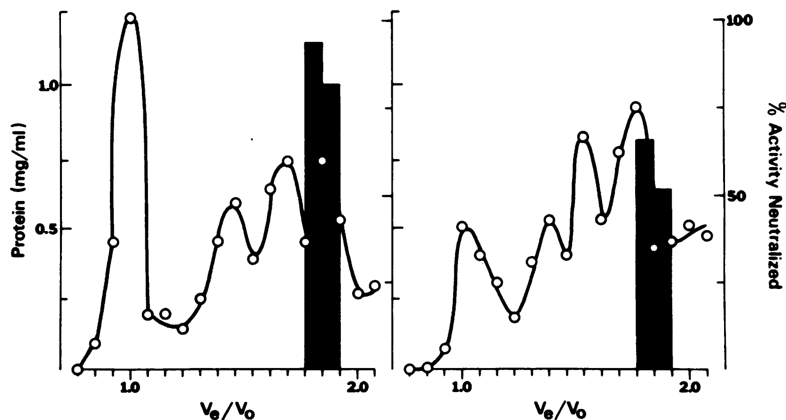

FIG. 5. *BioGel A 1.5 elution profile of receptor activity in sonic extracts from wild-type (left) and mutant (right) cells. Protein in sonic extract, after concentration by $(NH_4)_2SO_4$ precipitation, was fractionated by applying 0.7 ml of concentrate (18 mg of protein) to a column (1.5 by 50 cm) equilibrated in 0.2 M phosphate buffer; 2.25-ml fractions were collected at a flow rate of 10 ml/h. A 0.25-ml portion from each fraction was used to assay for mutacin-neutralizing activity (shaded areas) as described in the legend of Fig. 3. V_e, Elution volume; V_0, void volume.*

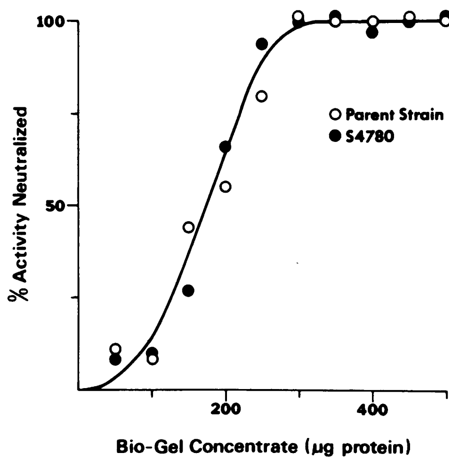

FIG. 6. *Mutacin-neutralizing activity of column-purified receptor. Fractions containing receptor activity (shaded area in Fig. 5) were pooled, and diluted portions were assayed for mutacin-neutralizing activity as described in the legend of Fig. 3.*

mutacin even when incubation mixtures contained 5 mg of carbohydrate. Whereas this finding does not rule out a role for carbohydrate or teichoic acids at the cell surface, it appears that, in vitro, no component in these acid-soluble fractions interacts with the mutacin to alter its biological activity. When receptor protein (0.5 mg) and trichloroacetic acid extract from mutant cells (1 to 2 mg) were preincubated before the addition of mutacin in the neutralization assay, no significant differences could be discerned from control mixtures (lacking trichloroacetic acid extracts). This observation suggests that in vitro the trichloroacetic acid-soluble material does not interfere with receptor-mediated neutralization of the mutacin.

DISCUSSION

Mutant S4780 adsorbs detectable amounts of mutacin GS-5 and possesses functional receptor protein, but yet is not killed by the bacteriocin at multiplicities that are almost two orders of magnitude greater than quantities lethal for wild-type cells. It would seem, therefore, that according to the classification conventionalized for gram-negative bacteria (11) and in at least one instance adopted for mutants of gram-positive organisms (9), S4780 should be considered as an example of the bacteriocin-tolerant phenotype. On the other hand, less than 20% of the mutacin apparently required to saturate adsorption sites in wild-type cells is neutralized by the mutant, suggesting that although the S4780 phenotype may not be equivalent to an extreme "receptor-minus" surface configuration, the disparate amount of mutacin adsorbed may be the causal basis for cell survival. If this is the case, then S4780 is an example of adsorption-defective variants which have been designated as resistant mutants (11). A third possibility, particularly interesting because several rounds of selection were required to obtain such a mutant, is that the phenotype exemplified by S4780 is a combination of changes, some of which include reduced surface density of receptor as well as abnormalities involving other moieties affected by mutacin-cell interaction.

All of the seven other isolates capable of growing in liquid media containing the mutacin at 80 KU per cell adsorbed as much bacteriocin as the parent strain. This observation, together with other features (which will be detailed in separate communications), suggests that the isolates conform to the commonly accepted definition of tolerance. When a number of the isolates capable of growing only at lower bacteriocin concentrations (20 to 40 KU per cell) were examined for their mutacin-neutralizing capacity, 11 out of 14 were found to reflect this selective bias towards the tolerant phenotype. The significance of this is unclear, but it is noteworthy that when selection procedures similar to one used herein have been adopted for the selection of colicin-insensitive cells, there is usually a preponderance of receptorless or resistant mutants (8).

I have not yet obtained a group A streptophage that will plate on the parent strain (MJP-2) used in this study and therefore am unable to determine whether phage and bacteriocin sensitivity are related. There are several well-documented examples of such sensitivity in coliform bacteria (3, 10).

ACKNOWLEDGMENTS

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