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Genetic Analysis of *Staphylococcus aureus* RNA Polymerase Mutants

T. O. MORROW,†* AND S. A. HARMON‡

Department of Biological Sciences, Bowling Green State University, Bowling Green, Ohio 43402

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Spontaneous mutants of *Staphylococcus aureus* resistant to rifampin, rifamycin SV, streptovaricin, or streptolydigin were isolated and shown to be resistant due to chromosomal rather than plasmid mutations. Based on data concerning spontaneous mutation rates, genetic cotransduction rates, and in vitro sensitivity studies, four major antibiotic cross-resistance patterns were found. The genetic markers responsible for these cross-resistance patterns were shown to be separable by transduction. Nonpurified RNA polymerase activity in lysates of mutants showed the same sensitivity to these antibiotics as shown by the mutants on solid media. A model is proposed explaining possible structure-function relationships involved in the binding of these antibiotics to the RNA polymerase molecule and the mutations resulting in resistance to these antibiotics. This model includes generally overlapping but different-sized binding sites on the RNA polymerase protein coded for by similarly arranged mutable sites on the DNA.

Rifampin and the related antibiotics, rifamycin SV, streptovaricin, and streptolydigin, have proven to be useful in the study of procaryotic RNA polymerase. Studies in vitro have shown that these four antibiotics bind to RNA polymerase molecules and inhibit their activity.

In *Escherichia coli*, rifampin, rifamycin SV (26), and streptovaricin (17) inhibit RNA polymerase from initiating transcription. Streptolydigin inhibits the elongation step of transcription in *E. coli* (5) and *Bacillus megaterium* (21). In *E. coli*, the β subunit of the RNA polymerase is altered in mutants resistant to rifampin and streptolydigin (10, 21) and it is thought that streptovaricin also affects the β subunit (10). In *Staphylococcus aureus*, it has also been shown that rifampin affects RNA polymerase activity (25). Mutants resistant to some of these antibiotics have been isolated in *E. coli* (16, 27), *B. subtilis* (8), and *Neisseria gonorrhoeae* (20). Mapping data from these mutants indicate linkage of the markers involved in resistance to the rifampin class of antibiotics. Resistances to rifampin and streptovaricin are generally more closely linked to each other than either marker is to streptolydigin resistance.

We have isolated chromosomal mutants of *S. aureus* which are resistant to rifampin, rifamycin SV, streptovaracin, and streptolydigin. Spontaneous mutation rates and cross-resistance patterns were determined for each mutant, and the mutants were mapped by transduction by using *S. aureus* bacteriophage 83. Four major antibiotic cross-resistance patterns were found, including a new combination in a mutant resistant to rifamycin SV and streptovaricin but sensitive to rifampin. The crude RNA polymerase activity in lysates of mutants showed the same sensitivity to these antibiotics as found for the mutant cells on solid media.

Recently, there has been an effort to standardize the many genotype symbols used for β subunit mutants (9). Due to the many cross-resistance patterns obtained in the study, the traditional *rif* (rifampin), *rsv* (rifamycin SV) *stv* (streptovaricin), and *stl* (streptolydigin) genotypes have been retained for clearer understanding when the genotypes of mutants are compared to phenotypes. In addition, however, *rpoB* (RNA polymerase β subunit) genotypes have been included in an effort to aid in standardization. R. S. Hayward has kindly assigned the first 100 *rpoB* genotypes in *S. aureus* to this laboratory.

MATERIALS AND METHODS

Bacterial strains and bacteriophage. Either *S. aureus* strain TM 655 or strain TM 656, a variant of TM 655 which lacks the plasmid for penicillinase production, was the parent of all the antibiotic-resistant strains used in this study. TM 655 was originally obtained from J. N. Baldwin and is of the same origin as organisms reported in the literature as strain 655 and as ISP 200. TM 656 was isolated by one of us (Harmon) in 1962. Table 1 lists the parent and mutant

† Present address: Biology Department, Clarion State College, Clarion, PA 16214.

‡ Present address: Veterans Administration Hospital, Salt Lake City, UT 84148.

TABLE 1. *S. aureus strains used in study*

Strain	Previous genotype[a]	Standardized genotype	Phenotype				Origin[b]
			Rif	Rsv	Stv	Stl	
TM 655			S	S	S	S	This laboratory
TM 656			S	S	S	S	This laboratory
TM 97	*rif-1*	*rpoB1*	R	R	R	S	TM 655
TM 100	*rif-4*	*rpoB2*	R	R	R	S	TM 655
TM 111	*rif-33*	*rpoB3*	R	R	R	R	TM 655
TM 113	*rif-1 stl-1*	*rpoB1 rpoB4*	R	R	R	R	TM 97
TM 125	*rsv-1*[c]	*rpoB5*	R	R	R	S	TM 655
TM 128	*rsv-19*	*rpoB6*	S	R	R	S	TM 655
TM 130	*rsv-29*	*rpoB7*	S	R	R	S	TM 655
TM 136	*rsv-19 stl-1*	*rpoB6 rpoB9*	S	R	R	R	TM 128
TM 147	*rsv-19 stl-1 rif-1*	*rpoB6 rpoB9 rpoB10*	R	R	R	R	TM 136
TM 150	*stv-35*	*rpoB11*	S	S	R	S	TM 655
TM 153	*stv-35 rsv-1*	*rpoB11 rpoB12*	S	R	R	S	TM 150
TM 155	*stv-35 rsv-1 rif-1*	*rpoB11 rpoB12 rpoB13*	R	R	R	S	TM 153
TM 158	*stv-35 rsv-1 rif-2*	*rpoB11 rpoB12 rpoB31*	R	R	R	S	TM 153
TM 165	*stv-35 rsv-1 rif-1 stl-1*	*rpoB11 rpoB12 rpoB13 rpoB14*	R	R	R	R	TM 153 after two independent selection steps on rifampin and streptolydigin, respectively
TM 168	*stv-35 rsv-2*	*rpoB11 rpoB15*	S	R	R	S	TM 150
TM 177	*stv-35 rsv-6 rif-6 stl-2*	*rpoB11 rpoB16 rpoB17 rpoB18*	R	R	R	R	TM 153 after three independent selection steps on rifamycin SV, rifampin, and streptolydigin, respectively
TM 179	*stv-35 rif-4*	*rpoB11 rpoB19*	R	R	R	S	TM 150
TM 181	*stv-35 rif-5*	*rpoB11 rpoB20*	R	R	R	S	TM 150
TM 184	*stv-35 stl-3*	*rpoB11 rpoB21*	S	S	R	R	TM 150
TM 192	*stl-1*	*rpoB22*	S	S	S	R	TM 655
TM 197	*stl-1 stv-1*	*rpoB22 rpoB23*	S	R	R	R	TM 192
TM 198	*stl-1 stv-2*	*rpoB22 rpoB24*	R	R	R	R	TM 192
TM 212	*stl-1 rsv-1*	*rpoB22 rpoB25*	R	R	R	R	TM 192
TM 215	*stl-2*	*rpoB26*	S	S	S	R	TM 655
TM 219	*stl-2 stv-2*	*rpoB26 rpoB27*	S	S	R	R	TM 215
TM 231	*stl-2 rif-1*	*rpoB26 rpoB28*	R	R	R	R	TM 215
TM 242	*stl-1 stv-2 rsv-1*	*rpoB22 rpoB24 rpoB29*	S	R	R	R	TM 219
TM 250	*stl-1 stv-2 rsv-1 rif-1*	*rpoB22 rpoB24 rpoB29 rpoB30*	R	R	R	R	TM 242

[a] Based upon antibiotic used for isolation step. Some may be resistant to other antibiotics due to cross-resistance.

[b] Strain indicated is that from which a spontaneous mutant was derived.

[c] *rsv*, New genotype to represent resistance to rifamycin SV.

strains, their derivations, and the patterns of resistance to the four antibiotics. All of the mutants were derived in this laboratory. *S. aureus* bacteriophage 83 was used for all transduction procedures.

Bacteria were routinely grown and stored on brain heart infusion agar (BHIA; Difco Laboratories) or in Trypticase soy broth (TSB; Baltimore Biological Laboratory [BBL]). Bacterial stocks were maintained on BHIA slants at −20 or 4°C. Phage were propagated by a modification of the agar overlay procedure of Pattee and Baldwin (18) and were maintained in TSB in sterile screw-capped tubes at 4°C.

Antibiotics. Rifampin, rifamycin SV, and streptovaricin were dissolved in distilled water to produce 100-μg/ml stock solutions, filter sterilized, and stored at −15°C. The solutions were thawed when needed and aseptically added to sterile media cooled to 50°C. Streptolydigin (free acid powder) was dissolved in a small amount of 95% ethanol and added directly to the sterile cooled media.

Determination of MICs. Minimal inhibitory concentrations (MICs) for each antibiotic were determined in TSB and on BHIA. Triplicate 10-tube doubling dilutions of the antibiotics were made in TSB. Antibiotic concentrations in the tubes ranged as follows (in micrograms per milliliter): rifampin and rifamycin SV, 1.0 to 0.00196; streptovaricin, 5 to 0.0196; and streptolydigin, 70 to 0.125. All tubes were inoculated with approximately 10^2 cells and incubated at 35°C for 24 h. End points were determined by a lack

of turbidity when the tubes were visually compared to uninoculated TSB. Determination of MICs on BHIA was done by preparing a series of plates containing decreasing amounts of the antibiotics and inoculating them with approximately 10^2 cells and incubating them for 24 h at 35°C. Once a general range for the MIC was determined, another series of plates with a narrower range of antibiotic concentrations was prepared and inoculated and incubated in a similar manner. End points were determined by the absence of macroscopically visible colonies.

Mutant isolation. All mutants used in this study were spontaneously occurring mutants, isolated by plating approximately 10^9 cells on BHIA containing the proper antibiotic at a relatively high concentration (in micrograms per milliliter: rifampin, 1; rifamycin SV, 1.5; streptovaricin, 7; and streptolydigin, 70). To insure independent derivation of mutant strains, each selection plate was seeded with the progeny of an independent colony, and only one mutant colony was taken from each selection plate.

After isolation, mutants were either replicated (14) or transferred by sterile toothpicks to three sets of BHIA, each containing one of the three antibiotics that was not used for the original isolation of the mutant. These plates were incubated at 35°C for 24 h and compared with each other to determine their cross-resistance to the other three antibiotics. All mutants were then cultured several times on BHIA containing the appropriate antibiotic(s) and stored on BHIA slants at 4°C. This technique was used two, three, or four times in succession to isolate the multiple mutation step mutants listed in Table 1. Mutants were assigned genotype designations indicative of the antibiotic(s) used in the isolation step(s) for obtaining that mutant. Due to different cross-resistance patterns, mutants named in this manner often had phenotypes not reflected in the genotype designation. For example, the previous genotype designation for TM 97 is *rif-1* because it was isolated on rifampin. This genotype designation does not reflect the fact that the mutant is also resistant to rifamycin SV and streptovaricin. Data concerning these mutants and their isolation are listed in Table 1.

Determination of mutation rates. Rates of mutation to resistance were determined for each of the four antibiotics. A total of 20 independent TSB cultures of strain TM 655 were used for each antibiotic. Each TSB tube was inoculated with approximately 10^2 cells and allowed to grow for 24 h at 35°C. Each inoculum was derived from an independent colony. Each culture was then plated in duplicate on BHIA plates which contained the appropriate antibiotic at the same concentration as described for mutant isolation. The plates were incubated for 24 h at 35°C, and the number of mutants was counted. To determine how many cells were placed on each antibiotic-containing plate, cell counts were done on BHIA plates which contained no antibiotic. The mutation rate was calculated by the zero class calculation method of Luria and Delbrück (15).

Crude RNA polymerase isolation and assay. The in vitro crude RNA polymerase activities of strain TM 655 and several of the mutants were assayed in the presence and absence of these antibiotics to determine whether mutations resulting in the cell's resistance to these antibiotics also resulted in a similar resistance for the crude RNA polymerase in vitro.

Cells were grown overnight at 35°C with shaking in TSB supplemented with 0.25% yeast extract (Difco). Two liters of TSB yielded approximately 10 g (wet weight) of log-phase cells. The cells were centrifuged at 10,000 × *g* for 15 min and suspended in 40 ml of tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.9). The buffer contained 50 mM Tris-hydrochloride (pH 7.9), 5 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.1 mM ethylenediaminetetraacetic acid, and 5% glycerol (vol/vol). Lysostaphin (Mead Johnson & Company, Evansville, Ind.) was added at a final concentration of 15 U/ml for 1 h at room temperature with stirring. The viscous mixture, cooled in a salt-ice mixture, was then subjected to sonic treatment with a Biosonik Sonifier (Bronwill Scientific Company, Rochester, N.Y.) at maximum output for 2 min.

The lysate was centrifuged at 14,000 × *g* for 15 min at 4°C, and the supernatant fluid was centrifuged at 105,000 × *g* for 120 min at 4°C in a Beckman L-1 preparative ultracentrifuge by using a type 30 rotor (Beckman Instruments, Palo Alto, Calif.). The ribosomal pellet was suspended in 10 ml of Tris-hydrochloride buffer which contained 0.5 M KCl. The suspended ribosomes were recentrifuged at 105,000 × *g* for 120 min at 4°C. The last two supernatants were combined to produce the ribosomal wash which was used for the assays.

The assay tubes (12 by 77 mm) were Siliclad (Clay Adams, New York, N.Y.) and contained a final volume of 0.3 ml including: 40 mM Tris-hydrochloride (pH 7.9); 10 mM $MgCl_2$; 0.1 mM ethylenediaminetetraacetic acid; 0.1 mM dithiothreitol; 0.15 M KCl; 0.15 mM UTP (sodium salt; Sigma Chemical Co., St. Louis, Mo.); 0.15 mM GTP (sodium salt; Sigma); 0.15 mM CTP (sodium salt; Sigma); 0.35 mM ATP (disodium salt; Sigma); and 0.035 mg of DNA (calf thymus; Sigma). A 6-μCi amount of [^{3}H]CTP (specific activity, 28.6 Ci/mmol; New England Nuclear Corp., Boston, Mass.) were also added to each tube. Antibiotics, when used, were added to give the following final concentrations: rifampin, rifamycin SV, and streptovaricin, 10 μg/ml; and streptolydigin, 100 μg/ml. Ribosomal wash material contained between 0.50 and 1.10 μg of protein per ml as determined by the procedure of Layne (13). The order of addition of reagents to the cold assay tubes was buffer, labeled and unlabeled nucleotides plus diluent as a mixture, then protein, and, finally, DNA. If an antibiotic was being used, it was preincubated with the ribosomal wash protein at 37°C for 30 s before it was added to the assay tube. All assays were done in duplicate.

The assay mixtures were incubated at 37°C for 20 min and chilled in an ice bath, and the contents were then pipetted onto Whatman 3 MM, 1-inch (ca. 2.54-cm) filter paper squares which were dried, and each was treated as follows: 20 min in 5 ml of cold 10% trichloroacetic acid, 10 min in 5 ml of cold 5% trichloroacetic acid, 10 min in 5 ml of cold, fresh 5% trichloroacetic acid, and 30 min in 5 ml of a 1:1 cold ether/ethanol solution. All trichloroacetic acid solutions contained 0.01 M sodium pyrophosphate. The

processed papers were air dried and counted for 10,000 counts in a model 3375 Packard liquid scintillation spectrometer (Packard Instrument Company, Downer's Grove, Ind.) in a dioxane-based scintillation cocktail. Controls included assay tubes lacking DNA or protein. Counts in the control tubes were subtracted from the counts in the corresponding experimental tubes.

Determination of whether resistance markers are chromosomal or extrachromosomal. Since many antibiotic resistance markers in *S. aureus* are plasmid borne, experiments were done to determine whether these resistance markers have a plasmid or chromosomal location. The effects of UV irradiation of donor phage on transduction rates have been used to determine the location of markers in *S. aureus*. Decreased transduction rates indicate plasmid locations while increased transduction rates indicate chromosomal locations (1, 2, 19). A transducing phage propagated on a rifampin-resistant mutant was UV irradiated for 120 s at a distance of 22 cm. Portions were removed after 30, 60, and 120 s, part of each portion was titrated for active phage, and part was used for transduction of a rifampin resistance marker to strain TM 655.

Transduction procedures. Transductants could not be isolated by the direct plating of the recipient cell–donor phage mixture on antibiotic-containing solid media, so the procedures of Pattee and Baldwin (18) were modified in several ways. For most experiments, the donor phage was irradiated for 1 min by using shortwave UV light at a distance of 22 cm before the phage were mixed with the recipient cells, and the transduction mixture was spread on plates of BHIA which contained 0.5% sodium citrate and no antibiotics. After incubation for 1 to 5 h at 35°C, 10-ml overlays of BHIA containing the antibiotic to be tested were poured over the plates. Antibiotic concentrations in the overlays were (in micrograms per milliliter) rifampin, 1; rifamycin SV, 1.5; streptovaricin, 7; and streptolydigin, 70.

When multiply resistant strains were used as donors, an analysis of unselected markers was done either by replica plating or by the sterile-toothpick transfer technique. Selection was on BHIA containing the same antibiotic concentrations as listed previously for the overlays.

RESULTS

MICs. The MICs of these four antibiotics in TSB and on BHIA plates are shown in Table 2. In general, MICs for each antibiotic were lower in broth than on solid media. In both types of media, rifampin had the lowest MIC, followed by rifamycin SV, streptovaricin, and streptolydigin, respectively.

Mutation rates. Table 3 presents the data on spontaneous mutation rates resulting in resistance to each of these antibiotics. Resistance to streptolydigin showed the highest mutation rate, followed by streptovaricin, rifamycin SV, and rifampin, respectively.

Isolation of mutants and analysis of cross-resistance patterns. A total of 191 independent mutants isolated on rifampin, 121 independent mutants isolated on rifamycin SV, 142 independent mutants isolated on streptovaricin, and 30 independent mutants isolated on streptolydigin were tested for cross-resistance to the three antibiotics which were not used for the initial selection. The cross-resistance patterns are shown in Table 4. Generally, mutants isolated on rifampin were also resistant to rifamycin SV and streptovaricin. Generally, mutants isolated on rifamycin SV were either also resistant to rifampin and streptovaricin or also resistant to streptovaricin. Generally, mutants isolated on streptovaricin were also resistant to rifampin, rifamycin SV, or also resistant to rifamycin SV, or resistant only to streptovaricin. Generally, mutants isolated on streptolydigin were not resistant to any of the other three antibiotics. The same basic resistance patterns were obtained whether strain TM 655 or strain TM 656 was used to obtain mutants. Some of these mutants were used to produce the multiple-mutation step mutants. For example, strain TM 150, which was initially isolated for resistance to streptovaricin, was not resistant to any of the other three antibiotics after the first selection step. It was then used to select a mutant also resistant to rifamycin SV, but not to rifampin or streptolydigin. This two-mutation step, doubly resistant mutant was then plated on media containing rifampin to select for resistance to rifampin. Then, finally, this three-mutation step, triply resistant mutant was plated on media containing streptolydigin

TABLE 2. *S. aureus strain TM 655, MICs of rifampin, rifamycin SV, streptovaricin, and streptolydigin in TSB and on BHIA*

Antibiotic	MIC (μg/ml)	
	TSB	BHIA
Rifampin	0.002	0.020
Rifamycin SV	0.031	0.10
Streptovaricin	0.313	4.20
Streptolydigin	17.40	30.00

TABLE 3. *Mutation rates for resistance to rifampin, rifamycin SV, streptovaricin, and streptolydigin in strain TM 655*

Antibiotic	Mutation rate[a]
Rifampin	4.57×10^{-8}
Rifamycin SV	6.20×10^{-8}
Streptovaricin	2.21×10^{-7}
Streptolydigin	1.04×10^{-7}

[a] Each value indicates the number of mutants per cell generation.

to produce a four-mutation step, quadruplicately resistant mutant. The mutants of these types which were used for this study are listed in Table 1.

In vitro effect of the antibiotics upon the crude RNA polymerase isolated from various mutants. Table 5 summarizes the results of the in vitro resistance patterns of the ribosomal washes of several of the mutants used in the study. The in vitro resistance patterns shown by the ribosomal washes of the mutants corresponded to the resistance patterns of the cell on solid media containing the various antibiotics. For example, the ribosomal wash from TM 100 was able to incorporate radioactive CTP in the presence of rifampin, rifamycin SV, and streptovaricin, but not in the presence of streptolydigin. This was the same pattern of resistance shown by the TM 100 cells on solid media containing these antibiotics, since it was able to grow on media containing rifampin, rifamycin SV, and streptovaricin, but not on media containing streptolydigin.

Chromosomal or plasmid location for markers responsible for resistance to rifampin. Figure 1 shows the effects of UV irradiation on donor phage used for transduction. With increasing time of irradiation the phage titer decreased and the transduction rate increased, indicating that the genetic markers for resistance to rifampin, and by association the rest of the markers in this study, are located on the chromosome rather than on a plasmid.

Transduction analysis of a number of single-step and multiple-step resistance mutants. Sixty different mutants resistant to different combinations of these antibiotics, some isolated in one step and others in as many as four steps (Table 1), were used as donors for transduction of resistance markers to strain TM 655. Transductants were independently selected on all antibiotics to which the donor was resist-

TABLE 4. *Cross-resistance patterns of 484 independently derived mutants*

Parent strain	Antibiotic used for isolation	Total no. of independent mutants tested	No. of mutants showing the following resistance patterns (phenotypes)						
			Rif^r Rsv^r Stv^r Stl^r	Rif^r Rsv^r Stv^r Stl^s	Rif^s Rsv^r Stv^r Stl^s	Rif^s Rsv^s Stv^r Stl^s	Rif^s Rsv^s Stv^r Stl^r	Rif^s Rsv^r Stv^r Stl^r	Rif^s Rsv^s Stv^s Stl^r
TM 655	Rifampin	104	1	103	—	—	—	—	—
TM 656	Rifampin	87	—[a]	87	—	—	—	—	—
TM 655	Rifamycin SV	50	1	37	12	—	—	—	—
TM 656	Rifamycin SV	71	—	57	14	—	—	—	—
TM 655	Streptovaricin	70	—	35	19	16	—	—	—
TM 656	Streptovaricin	72	—	33	22	17	—	—	—
TM 655	Streptolydigin	25	1	—	—	—	2	—	22
TM 656	Streptolydigin	5	—	—	—	—	—	—	5

[a] —, No mutants in the class.

TABLE 5. *In vitro resistance patterns of crude RNA polymerase isolated from strain 655 and several mutants resistant to one or more antibiotics*

Strain	Protein (mg/assay tube)	Avg cpm incorporated into trichloroacetic acid-insoluble material in the presence of:[a]				
		No antibiotics	Rifampin	Rifamycin SV	Streptovaricin	Streptolydigin
TM 655	1.10	285	0	0	0	0
TM 100	0.93	215	350	250	190	0
TM 111	1.03	400	350	435	600	610
TM 130	1.00	350	0	350	325	0
TM 150	0.52	175	0	0	125	0
TM 155	0.88	162	150	129	210	0
TM 158	0.58	220	300	150	153	0
TM 165	0.50	150	200	200	125	120
TM 192	0.92	240	15	10	0	260

[a] Values represent counts per minute (corrected for counts in controls). Control values were generally in the range of 1,000 cpm.

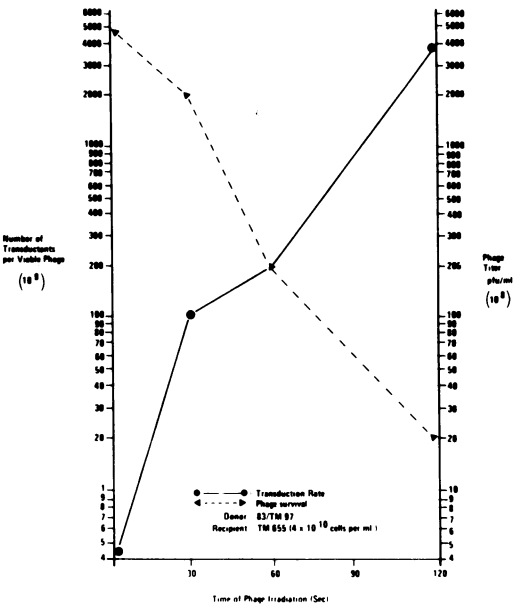

FIG. 1. *Effect of UV irradiation of phage on transduction of rifampin resistance.*

ant and then were analyzed for unselected markers by toothpick transfer to media containing the antibiotics not used in the initial selection. The transductional analysis of 20 representative mutants (Table 6), indicates that the mutations resulting in resistance to the antibiotics are separable by recombination.

DISCUSSION

Before mutants resistant to these antibiotics could be isolated, it was necessary to determine the concentration required to inhibit the growth of strain TM 655. The MICs for solid and liquid media (Table 2) correlated reasonably well with results previously described for other strains of *S. aureus* (3, 4, 7, 22). The MICs on solid media were higher than these in broth.

The various antibiotic concentrations in the media used to select mutants were considerably higher than the MICs for solid media. It was found that resistance to these antibiotics was an all-or-none phenomenon. For example, mutants resistant to 1 μg of rifampin per ml were also resistant to 100 μg/ml. This phenomenon was observed with all four antibiotics. Tsukamura observed similar results with *Mycobacterium tuberculosis* (24).

All mutants isolated in this study were spontaneous mutants. The mutation rates were high enough that mutagens did not have to be used (Table 3). Usually, plating 10^9 cells on an antibiotic-containing plate was sufficient to obtain 10 to 100 mutants.

The mutants selected for further study were assumed to be RNA polymerase mutants rather than permeability mutants, since the crude RNA polymerase activity of lysates made from mutants showed the same resistance patterns as the mutant cells did on solid media (Table 5). It is not possible to say positively that the mutants not tested in this manner were also RNA polymerase mutants, but transduction data and general growth characteristics of the mutants strongly suggest that none of the mutants used in the study were permeability mutants. Also, even though there is some reference in the literature to permeability mutants in *B. subtilis* (L. R. Brown and R. H. Doi, Bacteriol. Proc., p. 131, 1970) and *M. tuberculosis* (11), practically all others mentioned are RNA polymerase mutants. Crude RNA polymerase activity in two of the mutants tested (TM 100 and TM 111) seemed to be enhanced by one or more of the antibiotics. It is unclear whether this was an actual stimulation or a result of the variability of assays resulting from the use of crude enzyme preparations. The primary purpose of this experiment was to verify that the mutants were RNA polymerase mutants rather than permeability mutants and these results verify that they were RNA polymerase mutants.

These mutations all appeared to have chromosomal rather than plasmid locations. Increased transduction rates after irradiation of the donor phage with UV light has been shown to indicate chromosomal rather than plasmid markers (1, 2, 19). The plasmid of TM 655 carries a penicillinase marker which shows lower transduction rates when UV-irradiated phage are used. Figure 1 shows specific controlled data for one mutant, and UV irradiation routinely increased the rates of all transductions.

Some information concerning the nature of the genetic relationships between the mutations resulting in resistance to the four antibiotics was gained by analyzing the cross-resistance patterns of the mutants. Since these antibiotics are similar in their structure and mode of action, it is reasonable to believe there would be cross-resistance. Only certain specific cross-resistance patterns were observed for mutants isolated on each antibiotic (Table 4).

The four major resistance patterns which emerged were resistance to the streptovaricin alone (hereafter called a type 3 mutation), resistance to streptovaricin and rifamycin SV together (hereafter called a type 2 mutation), resistance to streptovaricin, rifamycin SV, and rifampin together (hereafter called a type 1 mu-

TABLE 6. *Transduction analysis using various resistant mutants as donors*[a]

Transduction no.	Donor strain	Antibiotic used for selection of transductants	No. of resistant transductants	% of transductants also resistant to:			
				Rifampin	Rifamycin SV	Streptovaricin	Streptolydigin
1	TM 100	Rifampin	131	—[b]	100	100	—
		Rifamycin SV	330	100	—	100	—
		Streptovaricin	1,088	100	100	—	—
2	TM 125	Rifampin	662	—	100	100	—
		Rifamycin SV	272	100	—	100	—
		Streptovaricin	102	100	100	—	—
3	TM 128	Rifamycin SV	140	—	—	100	—
		Streptovaricin	308	—	100	—	—
4	TM 113	Rifampin	136	—	100	100	82
		Rifamycin SV	176	100	—	100	88
		Streptovaricin	34	100	100	—	74
		Streptolydigin	98	93	93	93	—
5	TM 198	Rifampin	34	—	100	100	50
		Rifamycin SV	22	100	—	100	46
		Streptovaricin	50	100	100	—	70
		Streptolydigin	192	33	33	33	—
6	TM 212	Rifampin	149	—	100	100	52
		Rifamycin SV	73	100	—	100	53
		Streptovaricin	309	100	100	—	52
		Streptolydigin	340	49	49	49	—
7	TM 231	Rifampin	132	—	100	100	52
		Rifamycin SV	47	100	—	100	64
		Streptovaricin	196	100	100	—	64
		Streptolydigin	355	43	43	43	—
8	TM 153	Rifamycin SV	36	—	—	100	—
		Streptovaricin	126	—	91	—	—
9	TM 168	Rifamycin SV	88	—	—	100	—
		Streptovaricin	76	—	48	—	—
10	TM 179	Rifampin	78	—	100	100	—
		Rifamycin SV	150	100	—	100	—
		Streptovaricin	620	31	31	—	—
11	TM 181	Rifampin	332	—	100	100	—
		Rifamycin SV	230	100	—	100	—
		Streptovaricin	613	37	37	—	—
12	TM 184	Streptovaricin	104	—	—	—	56
		Streptolydigin	75	—	—	50	—
13	TM 219	Streptovaricin	53	—	—	—	45
		Streptolydigin	132	—	—	23	—
14	TM 136	Rifamycin SV	185	—	—	100	69
		Streptovaricin	128	—	100	—	65
		Streptolydigin	174	—	61	61	—
15	TM 197	Rifamycin SV	20	—	—	100	55
		Streptovaricin	156	—	100	—	60
		Streptolydigin	364	—	32	32	—
16	TM 147	Rifampin	221	—	100	100	54
		Rifamycin SV	173	100	—	100	52
		Streptovaricin	278	100	100	—	50
		Streptolydigin	188	53	53	53	—
17	TM 155	Rifampin	269	—	100	100	—
		Rifamycin SV	308	98	—	100	—
		Streptovaricin	215	97	97	—	—
18	TM 177	Rifampin	401	—	100	100	60
		Rifamycin SV	615	100	—	100	48
		Streptovaricin	502	98	99	—	61
		Streptolydigin	368	63	63	63	—
19	TM 242	Rifamycin SV	150	—	—	100	53
		Streptovaricin	230	—	98	—	47
		Streptolydigin	192	—	46	46	—
20	TM 250	Rifampin	156	—	100	100	47
		Rifamycin SV	131	100	—	100	51
		Streptovaricin	259	100	99	—	50
		Streptolydigin	202	48	48	48	—

[a] All transductions were done by using bacteriophage 83 as the transducing phage. Phage titers ranged from 3×10^9 plaque-forming units (PFU)/ml to 4.5×10^{11} PFU/ml. Recipient cells in all cases were strain TM 655. Cell concentration varied from 1.6×10^{10} cells/ml to 7.2×10^{10} cells/ml.

[b] —, Not replicated either because this was the same antibiotic used for the initial isolation of transductants or because the donor was not resistant to the antibiotic.

tation), and resistance to streptolydigin alone (hereafter called a type 4 mutation). Small numbers of mutants showing one of the first three patterns with the addition of streptolydigin resistance were also observed. Each of the four major patterns was theorized to represent a different alteration in the RNA polymerase molecule which affects the binding of these antibiotics to the enzyme.

Others have reported information concerning cross-resistance between these antibiotics, generally in organisms other than *S. aureus*. In *E. coli*, mutants resistant to streptovaricin and rifampin as well as a small number of mutants resistant only to streptovaricin were detected (27). In another study, a large number of *B. subtilis* rifampin mutants were all also resistant to streptovaricin, but not all mutants selected on streptovaricin were also resistant to rifampin (8). Streptolydigin was also used in this study (8), but no cross-resistance between streptolydigin and rifampin was detected. Another study (12) showed *E. coli* mutants selected on rifampin which were also resistant to streptolydigin. Mutants selected on streptovaricin became more, not less, sensitive to streptolydigin.

The cross-resistance patterns limited the steps which could be taken to create multiple-mutation step, multiply resistant mutants. For instance, all mutants selected on rifampin were type 1 mutants also resistant to rifamycin SV and streptovaricin. This meant it was impossible to use a rifampin-resistant mutant to select a streptovaricin- or rifamycin SV-resistant mutant. It was, however, possible to select mutants only resistant to streptovaricin (type 3 mutation) or streptolydigin (type 4 mutation), respectively. These patterns made it possible to produce four mutation step mutants resistant to all four antibiotics in two different sequences: (i) a type 3 mutation, followed by a type 2 mutation, followed by a type 1 mutation, followed by a type 4 mutation or (ii) a type 4 mutation, followed by a type 3 mutation, followed by a type 2 mutation, followed by a type 1 mutation. These sequences or parts of them were used to produce mutants to be used as donors in transductions, which could be used to see if these markers were separable by recombination.

A large number of transductions using various mutants as donors supported the theory that there are four genetically separable markers involved in this system. Results of some of these transductions are shown in Table 6. Transductions 1 and 2 simply demonstrated that type 1 mutations did convey resistance to rifampin, rifamycin SV, and streptovaricin. All transductants were resistant to all three antibiotics, regardless of which antibiotic was used for selection. Transduction 3 shows similar results for a type 2 mutation. Transductions 4, 5, 6, and 7 demonstrated that type 1 and 4 mutations were genetically distinct and separable by transduction showing cotransduction frequencies ranging from 33 to 93%. These were type 1 mutants selected on each of the antibiotics that a type 1 mutation produced resistance to. Throughout the transduction experiment data, there are a number of columns which show identical percentages of coresistance for various "unselected markers." For example, in transduction 4, 93% of the transductants selected on streptolydigin were also resistant to rifampin, rifamycin SV, and streptovaricin. These were the same colonies showing resistance to these three antibiotics. In this case, this demonstrates the presence of a type 1 marker. When similar identical percentages are shown in the data from other transduction experiments, the same principle applies.

Transductions 8 and 9 demonstrated that type 2 and type 3 mutations were genetically distinct and separable by transduction, showing cotransduction frequencies ranging from 48 to 91%. Transductions 10 and 11 demonstrated that type 1 and type 3 mutations were genetically distinct and separable by transduction showing cotransduction frequencies ranging from 31 to 37%. Transductions 12 and 13 demonstrated that type 3 and type 4 mutations were genetically distinct and separable by transduction showing cotransduction frequencies ranging from 23 to 50%. Transductions 14 and 15 demonstrated that type 2 and type 4 mutations were genetically distinct and separable by transduction showing cotransduction frequencies ranging from 32 to 61%. Transductions 16, 17, 18, 19, and 20 represent efforts to separate various 1-, 2-, 3-, and 4-type markers from each other when the donor possesses 3 or 4 of these markers. Generally, it was very difficult to separate type 1, 2, and 3 markers from each other when they were present in one of the multiple-mutation-step, multiply resistant donors. Cotransduction frequencies for these markers were in the 97 to 99% range. This makes it difficult to determine whether this is true cotransduction or simply a result of spontaneous mutants. The overlapping nature of resistance caused by these mutations probably makes separation by transduction very difficult when more than two of these markers are present in a particular donor.

The cross-resistance patterns, mutation rate data, and transduction results suggested a model for structural and genetic relationships between these various classes of mutants in *S. aureus*. These data suggested that there may be several

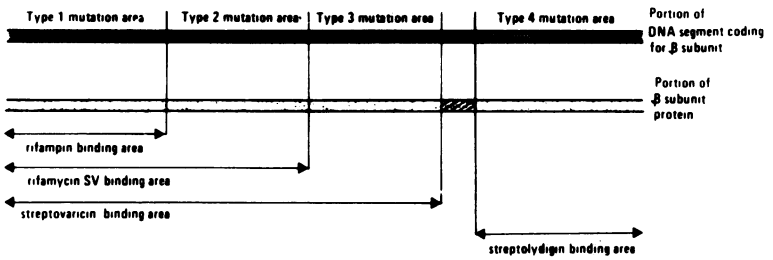

FIG. 2. *Schematic model for the correlation of the S. aureus mutations resulting in cross-resistance to rifampin, rifamycin SV, streptovaricin, and streptolydigin and the binding of the antibiotics to the β subunit protein of the RNA polymerase molecule. Actual relative length of each area may be different than presented.*

binding sites or different-sized binding sites for each of these antibiotics and that some of the binding sites overlapped. Figure 2 is a schematic representation of this theory. The numbered areas represent mutational sites on the DNA as well as the corresponding antibiotic binding areas on the RNA polymerase β subunit. An alteration in area 1 would yield a type 1 mutant resistant to rifampin, rifamycin SV, and streptovaricin. An alteration in any part of area 2 would yield a type 2 mutant resistant to rifamycin SV and streptovaricin, but not rifampin. An alteration in any part of area 3 would yield a type 3 mutant resistant only to streptovaricin. An alteration in any part of area 4 would yield a type 4 mutant resistant to streptolydigin only. The mutation rate data (Table 3) supported Fig. 2, in that the highest mutation rate occurred when streptovaricin was used for selection of mutants and the lower mutation rate occurred when rifampin was used for the selection of mutants. Although other factors, such as mutation "hot spots," could be involved, it is probable that a higher mutation rate indicates a larger section of DNA and, consequently, protein which can affect resistance (binding) to a particular antibiotic. Since resistance to streptolydigin generally did not occur in combination with resistance to the others, it is not possible to use mutation rates to assign a particular length to the streptolydigin-affected region. Also, this indicated that in *S. aureus* there is little or no overlap between the mutation/binding sites for streptolydigin and the other three antibiotics. This is in contrast to data reported for *E. coli* (12) which indicated that approximately 20% of mutants selected for resistance to rifampin were also resistant to streptolydigin.

The separation of the streptolydigin region from the other three is reasonable, since it has been shown to affect the elongation functions of the β subunit rather than the initiation functions affected by the other three (10). Whether the few mutants isolated which showed one of the first three major resistance patterns in combination with streptolydigin resistance are double mutants, large deletions, or actually represent overlap between mutable areas is unclear. It is doubtful that they are permeability mutants because one of the mutants of this type, TM 111, had an RNA polymerase which showed in vitro resistance to all four antibiotics.

Some recent work has been done with the RNA polymerase binding site for rifamycin in *E. coli* (23), and the existence of two different binding sites for different rifamycin SV antibiotics on T-7 RNA polymerase has been postulated by Chamberlin and Ring (6), supplying supportive evidence for a multiple-binding-site model.

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LITERATURE CITED

1. **Arber, W.** 1960. Transduction of chromosome genes and episomes in *Escherichia coli*. Virology **2**:273–288.
2. **Asheshov, E. H.** 1966. Chromosomal location of the genetic elements controlling penicillinase production in a strain of *Staphylococcus aureus*. Nature (London) **210**:804–806.
3. **Bergamini, N., and G. Fowst.** 1965. Rifamycin SV, a review. Arzneim. Forsch. **15**:951–1002.
4. **Binda, G. E., E. Domenichini, A. Gottardi, B. Orlandi, E. Ortelli, B. Pacini, and G. Fowst.** 1971. Rifampicin, a general review. Arzneim. Forsch. **21**:1907–1978.
5. **Cassani, G., R. R. Burgess, H. M. Goodman, and L. Gold.** 1971. Inhibition of RNA polymerase by streptolydigin. Nature (London) New Biol. **230**:197–200.
6. **Chamberlin, M., and J. Ring.** 1972. Characterization of T7 specific RNA polymerase-III. Inhibition by derivatives of rifamycin SV. Biochem. Biophys. Res. Commun. **49**:1129–1136.
7. **DeBoer, C., A. Dietz, W. S. Silver, and G. M. Savage.** 1956. Streptolydigin, a new antimicrobial antibiotic. I. Biologic studies of streptolydigin, p. 866–892. *In* H. Welch and F. Marti-Ibanez (ed.), Antibiotic annual (1955–1956). Medical Encyclopedia, Inc., New York.

8. **Haworth, S. R., and L. R. Brown.** 1973. Genetic analysis of ribonucleic acid polymerase mutants of *Bacillus subtilis*. J. Bacteriol. **114**:103–113.
9. **Hayward, R. S., and J. G. Scaife.** 1976. Systematic nomenclature for the RNA polymerase genes of prokaryotes. Nature (London) **260**:246–248.
10. **Heil, A., and W. Zillig.** 1970. Reconstitution of bacterial DNA-dependent RNA-polymerase from isolated subunits as a tool for the elucidation of the role of the subunits in transcription. FEBS Lett. **11**:165–168.
11. **Hui, J., N. Gordon, and R. Kajioka.** 1977. Permeability barrier to rifampin in mycobacteria. Antimicrob. Agents Chemother. **11**:773–779.
12. **Iwakura, Y., A. Ishihama, and T. Yura.** 1972. RNA polymerase mutants of *Escherichia coli*. II. Streptolydigin resistance and its relation to rifampicin resistance. Mol. Gen. Genet. **121**:181–196.
13. **Layne, E.** 1957. Spectrophotometric and turbidimetric methods for measuring proteins. Methods Enzymol. **3**: 451–454.
14. **Lederberg, J., and E. M. Lederberg.** 1952. Replica plating and indirect selection of bacterial mutants. J. Bacteriol. **63**:399–406.
15. **Luria, S. E., and M. Delbrück.** 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics **28**:491–511.
16. **Mindlin, S. Z., T. S. Ilyina, T. A. Vowykova, and V. V. Velkov.** 1972. Genetical analysis of rifampin resistant mutants of *E. coli* K-12. Mol. Gen. Genet. **115**: 115–121.
17. **Mizuno, D., H. Yamazaki, R. Nitta, and H. Umezawa.** 1968. Inhibition of initiation of DNA-dependent RNA synthesis by the antibiotic B44P. Biochem. Biophys. Res. Commun. **30**:379–385.
18. **Pattee, P. A., and J. N. Baldwin.** 1961. Transduction of resistance of chlortetracycline and novobiocin in *Staphylococcus aureus*. J. Bacteriol. **82**:875–881.
19. **Poston, S. M.** 1966. Cellular location of the genes controlling penicillinase production and resistance to streptomycin and tetracycline in a strain of *Staphylococcus aureus*. Nature (London) **210**:802–804.
20. **Sarubbi, F. A., Jr., E. Blackman, and P. F. Sparling.** 1974. Genetic mapping of linked antibiotic resistance loci in *Neisseria gonorrhoeae*. J. Bacteriol. **120**: 1284–1292.
21. **Siddhikol, C. J., W. Ebstoeszer, and B. Weisblum.** 1969. Mode of action of streptolydigin. J. Bacteriol. **99**: 151–155.
22. **Siminoff, P., R. M. Smith, W. T. Solkalski, and G. M. Savage.** 1957. Streptovaricin. I. Discovery and biologic activity. Am. Rev. Respir. Dis. **75**:576–583.
23. **Stender, W., and K. H. Scheit.** 1977. Studies of the topography of the binding site of DNA-dependent RNA polymerase from *Escherichia coli* for the antibiotic rifamycin SV. Eur. J. Biochem. **76**:591–600.
24. **Tsukamura, M.** 1972. The pattern of resistance development to rifampicin in *Mycobacterium tuberculosis*. Tubercle **53**:111–117.
25. **Wehrli, W., F. Knusel, K. Schmid, and M. Staehelin.** 1968. Action of rifamycin on RNA-polymerase from sensitive and resistant bacteria. Biochem. Biophys. Res. Commun. **32**:284–288.
26. **Wehrli, W., and M. Staehelin.** 1971. Actions of the rifamycins. Bacteriol. Rev. **35**:290–309.
27. **Yura, T., and K. Igarashi.** 1969. Molecular and genetic resistance to streptovaricins and rifamycins in *Escherichia coli*. Proc. 6th Int. Congr. Chemother, vol. 2, p. 405–409.